An Improved Sandwich ELISA Method for the Determination of Immunoreactive Schizophyllan (SPG)

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As it is important to determine the optimal serum concentration of schizophyllan (SPG) when it is used as an anti-cancer drug, we devised a solid-phase ELISA. We also developed a sandwich ELISA using murine anti-SPG monoclonal antibody as the first antibody and rabbit anti-SPG serum as the second antibody in order to improve the detection sensitivity. This assay was able to determine SPG concentrations over 1.0 ng/ml and the absorbance at 490 nm was directly proportional to the SPG concentration. SPG in rabbit serum, obtained after intravenous and intramuscular injection (SPG; 10 mg/kg), was determined by this sandwich ELISA. Furthermore, the sensitivity of this ELISA method was compared with that of the Limulus test. The Limulus test is able to detect SPG in physiological saline (pH 6.3) at concentrations greater than 1.0 μg/ml, but the sensitivity increased when SPG was dissolved in alkaline solution (pH 12.0), enabling SPG to be measured almost down to 1.0 ng/ml. These data suggest that our sandwich ELISA may be used for the measurement of SPG in blood or tissue.

Keywords schizophyllan; sandwich ELISA; Limulus test; polysaccharide

Schizophyllan (SPG), an antitumor polysaccharide, is produced extracellularly by Schizophyllum commune Fries.1 It consists of (1→3)-β-D-glucan with branching (1→6)-β-D-glucose residues,2 and displays host-mediated antitumor activity against a variety of tumors.3 It is known that SPG forms a triple helical structure in aqueous solution, and that its conformation changes in alkaline solution or dimethylsulfoxide.3 As SPG is used clinically for the treatment of uterine cervical cancer,4 it is of vital importance to determine the optimal SPG concentration in human serum in order to monitor the therapeutic efficacy.

It is known that Limulus amebocyte lysate reacts with lipopolysaccharide (LPS) and (1→3)-β-D-glucan5 and that the coagulation Factor G present in amebocyte lysate is related to the reactivity of (1→3)-β-D-glucan.6 It has been reported recently that (1→3)-β-D-glucans can be detected by the Limulus test, implying a structure–activity relationship.7 We have already succeeded in preparing a murine anti-SPG monoclonal antibody (SPG1-HS) by fusing X63-Ag8.6.5.3. mouse myeloma cells with spleen cells from a BALB/c mouse immunized with SPG.8 This monoclonal antibody (MAb) reacted with some (1→3)-β-D-glucans and it seemed likely that it recognized the (1→3)-β-linkage sites. Moreover, we have also prepared another monoclonal antibody (MPG2) which reacts with SPG and Maitake proteoglycan.9 We have reported previously the construction of a solid-phase ELISA using MAb SPG1-HS.10 Using this ELISA method, SPG concentrations greater than 1.0 μg/ml were detectable. In order to able to measure lower concentrations of SPG in serum, this method needed to be improved. In the present paper, we reported the development of an improved sandwich ELISA for determining the optimal concentration of SPG in serum and compare it with the Limulus test.

MATERIALS AND METHODS

Animals New Zealand white female rabbits were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan.

Chemicals SPG (Mw: 4.5 × 105) was prepared by Taito Co., Ltd. The molecular weight of the SPG was estimated from the viscosity–molecular weight relationship described previously.11 Sodium hydroxide was purchased from Nacalai Tesque, Inc., Kyoto, Japan. Water and physiological saline were purchased from Otsuka Pharmaceutical Co., Ltd., Japan.

Preparation of Rabbit Anti-SPG Serum SPG dissolved in physiological saline was emulsified with an equal volume of complete Freund’s adjuvant and injected into rabbits subcutaneously five times at 2-week intervals. The antiserum was purified by salting out with saturated ammonium sulfate and subsequent affinity chromatography (Affi-Gel Protein A MAPS II kit, Bio-Rad Co.) as described elsewhere.12,13

Preparation of MAb SPG1-HS MAb SPG1-HS was prepared and characterized as described previously.8

Sandwich Enzyme-Linked Immunosorbent Assay (Sandwich ELISA) Fifty microliters of purified MAb SPG1-HS (1 mg/ml, 1:20-diluted) was coated onto each well of a 96-well flat-bottomed ELISA plate (Sumitomo Bakelite) at room temperature (RT) for 4 h. After washing with phosphate-buffered saline (PBS) three times, 100 μl 2% porcine serum albumin was then added for blocking. The sample solution (50 μl/well) was added and reacted at RT for 1 h. After washing with PBS, 50 μl biotinylated purified rabbit anti-SPG serum (1 mg/ml, 1:10-diluted) was added as the secondary antibody and reacted for 1 h. After washing with PBS, 50 μl avidin–biotin–peroxidase complex (ABC-PO kit, Vectastain, Vector Labs., U.S.A.) was added and reacted for 45 min. After washing with PBS, a chromogenic solution (o-phenylenediamine, H2O2/0.05 M

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citrate buffer) was added and reacted for 15 min. The enzyme reaction was terminated by adding 2 m H$_2$SO$_4$ (50 μl/well), and then the absorbance at 490 nm was read using an ELISA autoreader.

**Limulus Test** An endotoxin quantitative kit (Toxicolor System LS-6 Set, Seikagaku Kogyo Co., Ltd., Tokyo, Japan) and a glucan quantitative kit (Glupac GS-6 Set, Seikagaku Kogyo Co., Ltd., Tokyo, Japan) were used for SPG measurement. Under LPS-free conditions, 25 μl of each sample was placed on an endotoxin-free 96-well flat-bottomed plate (Toxipet, Seikagaku Kogyo Co., Ltd., Tokyo, Japan). Then, the reagent (LS-6 or GS-6, 25 μl) was added to each well and reacted at 37°C for 30 min. After addition of the chromogenic solution, the absorbance at 570 nm was read.

**Preparation of Rabbit Serum Containing SPG** SPG/physiological saline (10 mg/10 ml) was injected into rabbits intramuscularly or intravenously. Then, blood was collected from an ear vein at 0, 1, 2, 3, 4, 5, 6, 24, 48, and 72 h after injection. After centrifugation at 3000 rpm for 15 min, the supernatants were collected and stored at −20°C before use. These rabbit sera were treated with perchloric acid to remove proteins as described elsewhere. The supernatants were neutralized or made alkaline using sodium hydroxide.

**RESULTS**

We have established a sandwich ELISA using polyclonal antibodies and MAb SPG1-HS to measure the concentration of an antitumor polysaccharide, SPG. Firstly, SPG in physiological saline (SPG concentration: 0, 100.0 pg/ml, 1.0, 10.0, 100.0 ng/ml, 1.0, 10.0 μg/ml) was used and a sandwich ELISA using MAb SPG1-HS as the first Ab was carried out to assess the sensitivity. This ELISA detected SPG at a concentration greater than 1.0 ng/ml, as shown in Fig. 1a. The absorbance at 490 nm in the sandwich ELISA was directly proportional to the SPG concentration. The final yield after the addition of 1.0 μg/ml SPG was over 90%. When SPG standard solutions, prepared as described above and diluted with fetal calf serum (FCS), were used, the sandwich ELISA was able to detect SPG with a similar sensitivity (data not shown). Figure 1b shows the sensitivity of the **Limulus** test (Toxicolor system LS-6 Set). In this assay, SPG standard solutions diluted with physiological saline (pH 6.3) were used, and SPG concentrations greater than 1.0 μg/ml could be measured. When SPG/FCS standard solutions treated with perchloric acid were used in the **Limulus** test, data comparable with the sandwich ELISA were obtained. As the SPG-specific conformation (triple helix) may affect the sensitivity of the **Limulus** test, the SPG standard solution was diluted with alkali to obtain single-stranded SPG. Figure 2 shows that the pH affected the sensitivity. The **Limulus** test detected SPG under alkaline conditions (pH 12.0) at concentrations greater than 1.0 ng/ml. Each sample thus prepared was adjusted to pH 12.0 after the addition of perchloric acid. Next, we measured the time course of the SPG concentrations in the sera of rabbits injected intravenously (i.v.) or intramuscularly (i.m.) with SPG. Figure 3 shows the relationship between time and the SPG concentration in rabbit sera. The samples were treated with perchloric acid and then the pH adjusted. Sandwich ELISA showed that intramuscular injection produced a rapid increase in the concentration of SPG, reaching a maximum (approximately 4.2 μg/ml) at 6 h after administration, thereafter decreasing gradually, as shown in Fig. 3a. The SPG was

![Fig. 1](image-url)  
Fig. 1. Relationship between SPG Concentration and Absorbance in the Sandwich ELISA (a) and **Limulus** Test (b)  
SPG was dissolved in and diluted with physiological saline solution (pH 6.3). Each value represents the mean ± S.D. of three independent experimental results after subtraction of the background.
still detectable 72 h after administration. After intravenous administration, the SPG concentration increased more rapidly than after intramuscular injection. The maximum concentration (approximately 2.4 μg/ml) was reached 6 h after administration and then decreased. When we used rabbit sera without perchloric acid treatment, SPG in the sera could be measured by the sandwich ELISA. Similar results were obtained using the Limulus test, as shown in Fig. 3b, but there was a slight difference in the SPG concentrations when the two routes of administration were compared (correlation coefficient \( r = 2.60 \)). The maximum concentration was reached 4 and 5 h after i.m. and i.v. administrations, respectively.

**DISCUSSION**

We have prepared several murine monoclonal antibodies capable of reacting with SPG, which can be used to detect SPG in blood or tissues.\(^8,^9\) A sandwich ELISA using these antibodies has been set up and found to be highly sensitive. The ELISA was able to measure the concentration of SPG in the serum of rabbits after it had been administered by intravenous and intramuscular injection. These results are consistent with the data obtained using radioisotope-labeled SPG.\(^1,^5\) The sensitivity of our sandwich ELISA was compared with that of the Limulus test, which is used generally to measure lipopolysaccharides (LPS).\(^5\) Under alkaline conditions, SPG could be measured with much greater sensitivity (about 10 times higher than under neutral conditions) by the Limulus test. It was confirmed that SPG with a triple helix was detected by the Limulus test, as well as single-stranded SPG, as reported by Yadomae et al.\(^7\) However, in the Limulus test, LPS have to be removed, so this is an important disadvantage. Also, treatment with perchloric acid\(^11\) is required when serum is used. In contrast, with the sandwich ELISA, it is not necessary to remove protein and the assay can be conducted in the presence of LPS. Furthermore, this sandwich ELISA is specific for SPG, whereas the Limulus test cross-reacts with both LPS and (1→3)-β-D-glucans.

SPG is known to be an antitumor polysaccharide\(^1^)\) and

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**Fig. 2.** Limulus Test Reactivity of SPG at pH 6.3 or pH 12.0

- ■, SPG was dissolved in H\(_2\)O and diluted with physiological saline (pH 6.3); — □ —, SPG was dissolved and diluted with 0.25 N sodium hydroxide (pH 12.0). Each value represents the mean of three independent experimental results after subtraction of the background.

**Fig. 3.** Plasma Concentration of SPG in the Rabbit Measured by the Sandwich ELISA (a) or Limulus Test (b)

- ▲ —, SPG (10 mg) was injected intravenously; — ▲ —, SPG (10 mg) was injected intramuscularly.
sulfated SPGs possess anti-human immunodeficiency virus (HIV) activity, as reported previously.\textsuperscript{16}) Also, it has been shown that sulfated SPGs and sulfated oligosaccharides are effective inhibitors of plasmin.\textsuperscript{17}) Some of the MAbs prepared previously also reacted with sulfated SPG (data not shown). So the sandwich ELISA may be able to detect these SPG derivatives.

In summary, although our sandwich ELISA and the *Limulus* test are both useful for the detection of SPG in blood or tissues, the ELISA is superior to the *Limulus* test in terms of sensitivity. Therefore, our method is expected to prove useful for immunological studies involving \(\beta\)-d-glucans.

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REFERENCES


