別紙様式第４

学　位　論　文　要　旨

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［論文題名］

Evaluation of LIPS (luciferase immunoprecipitation system) for serodiagnosis of Toxoplasmosis

トキソプラズマ症血清診断におけるルシフェラーゼ免疫沈降システム（リップス法）の評価

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［要　旨］

Abstract

Background

Toxoplasma gondii is a protozoan parasite that causes toxoplasmosis. It can infect all warm-blooded animals, including humans, and it affects up to one-third of the human population. Most toxoplasmosis cases are asymptomatic, but it can lead to significant morbidity and mortality in congenitally infected individuals and immunocompromised patients. Currently, serological tests are the main diagnostic methods for toxoplasmosis. The Sabin-Feldman dye test is the gold standard because of its sensitivity and specificity in humans. However, this test is not suitable for routine diagnosis because it requires highly skilled practitioners and live parasites. Tachyzoite lysate antigens (TLAs) used in the enzyme-linked immunosorbent assay (ELISA) can cross-react significantly with other microorganisms' antigens, leading to false-positive results. ELISA with TLAs is not capable of discriminating between acute and chronic infections, making it difficult to standardize. Recombinant *Toxoplasma* antigens are considered to be good replacements for crude TLAs; they can be easily produced in large amounts with consistent quality, potentially enabling their use in routine screening of toxoplasmosis in humans. Other immunoassays, such as immunosorbent agglutination assay (ISAGA), indirect immunofluorescence antibody test (IFAT), and western blot, are available, but not commonly performed.

Development of reliable, quantitative technologies for serodiagnosis of *Toxoplasma gondii* infection remains desirable. The luciferase immunoprecipitation system (LIPS) is a relatively simple, highly sensitive, and rapid quantitative immunoassay. The major

advantages of this assay over ELISA are a wider dynamic range, shorter overall assay time, and less sample volume. A number of parasitic infections, such as strongyloidiasis, onchocerciasis, and loiasis, have been diagnosed using LIPS, which showed improved performance when compared with ELISA. In this study, we aimed to use this technology for the serodiagnosis of toxoplasmosis with four *T. gondii* antigens, namely, GRA6, GRA7, GRA8, and BAG1.

Principle of the LIPS

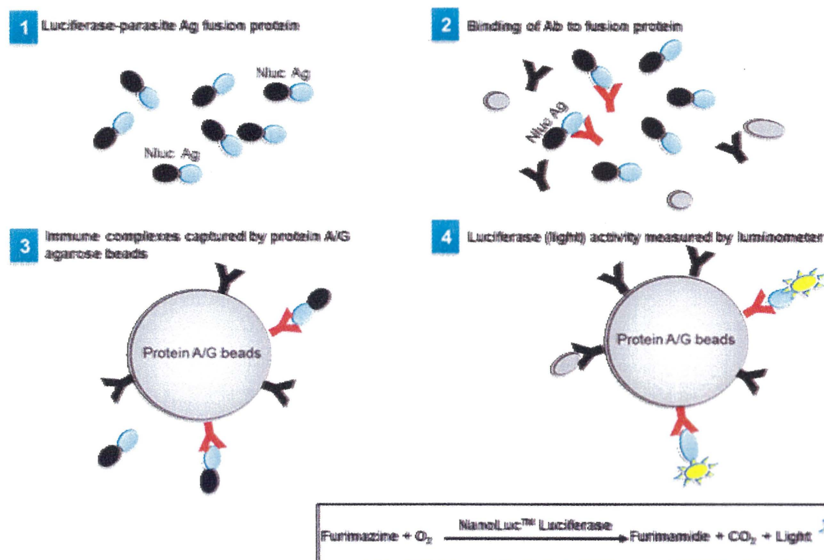

Fig. 1. Principle of the luciferase immunoprecipitation system (LIPS). Black oval shape indicates Nano-Luciferase (Nluc), and pale blue oval shape indicates the parasite antigen. Specific antibodies (shown as a Y-shape in red) bind to the parasite antigen and form immune complexes, which are then captured by protein A/G beads. Addition of furimazine substrate produces light, which is measured with a luminometer.

Methods

T. gondii (RH strain) tachyzoites were cultured in human fibroblast cell lines (HFF). The tachyzoites were used for the RNA preparation and cDNA synthesis. The tachyzoite cDNA was used as a PCR template to obtain DNA fragments that encode GRA6, GRA7, and GRA8. DNA fragments encoding BAG1 and nanoluciferase (Nluc, a small luciferase enzyme) were obtained by artificial gene synthesis instead of PCR.
Recombinant *Toxoplasma* antigens (dense granule antigens GRA6, GRA7, and GRA8 and bradyzoite antigen BAG1) fused with nanoluciferase (Nluc) were expressed in *Escherichia coli*, purified, and tested in LIPS assays with sera from mice experimentally infected with *T. gondii* and a WHO standard anti-*Toxoplasma* human

immunoglobulin (TOXM). The LIPS assays results were compared with conventional ELISA.

Results

A total of nine recombinant proteins were produced. Four were Nluc-*T. gondii* antigen fusion proteins (Nluc-GRA6, Nluc-GRA7, Nluc-GRA8, and Nluc-BAG1), four were *T. gondii* antigens without Nluc (GRA6, GRA7, GRA8, and BAG1), and one was Nluc. All recombinant proteins were produced as soluble proteins in *E. coli.* The patterns of reactivity, over the course of infection, differed from antigen to antigen. In the experimentally infected mice, LIPS assays detected antibodies against Nluc-GRA6, Nluc-GRA7, and Nluc-GRA8 as early as day 14, whereas antibodies against Nluc-BAG1 remained undetected until day 21 and then showed significant elevation on day 60. The same set of mouse sera were tested by conventional ELISA for comparison. In case of GRA6, the LIPS assay showed superior performance with clearer separation of RLU values for the uninfected (day 0) and infected (days 14, 21, and 60) samples. The LIPS assay with Nluc-BAG1 also showed better performance compared to ELISA on the basis of the clearer separation on day 60. In the case of GRA8, ELISA showed better performance for diagnosing the infection using day-21 sera (All six samples were diagnosed positive with ELISA, whereas three samples were judged negative with LIPS). In the case of GRA7, the performance of LIPS and ELISA appeared to be similar.

As for TOXM sera, LIPS assays with each Nluc recombinant protein produced reliable standard curves with a coefficient of determination (R^2) of 0.980–0.989 for GRA6, 0.986–0.990 for GRA7, 0.998–0.999 for GRA8, and 0.942–0.987 for BAG1. The detection limits were estimated to be 3.9, 2, 1, and 1 IU/ml for rGRA6, rGRA7, rGRA8, and rBAG1, respectively. The RLU values obtained with the immunoglobulin-depleted human serum were less than the values of the no-serum control.

To assess the specificities of each *T. gondii* LIPS, competition experiments with homologous and heterologous competitors were conducted. In each reaction with Nluc-*T. gondii* antigen and infected mice sera, increasing amounts of competitors were included. As expected, more RLU reductions were observed with homologous inhibitors than with heterologous ones. The specificity of our *T. gondii* LIPS was further assessed with sera from mice infected with some other parasitic pathogens, namely, *S. venezuelensis*, *T. canis*, *S. mansoni*, *M. vogae*, and *P. berghei*. The RLU values obtained with these sera were below the 3SD cut-off.

Discussion

In our study, antibodies against Nluc-GRA6, Nluc-GRA7, and Nluc-GRA8 could be detected within 14 days (ie. acute phase infection) by both LIPS and ELISA. The antibody level against BAG1 peaked significantly on only day 60 (during the chronic phase of infection), suggesting that anti-BAG1 antibodies may be useful as markers for chronic toxoplasmosis. A drawback of LIPS is the inability to detect IgM specifically. Protein A/G does not bind to murine IgM and shows only a weak binding to human IgM. Therefore, we speculate that most of the luciferase activities observed in the LIPS experiments with murine sera in the present study were attributable to the IgG bound to LIPS antigens.

To use LIPS technology to diagnose toxoplasmosis in humans, TOXM was tested in the LIPS assays with each recombinant fusion protein. The assays yielded good model fits to the data, with values of R^2 ranging from 0.942 to 0.999. Moreover, we found the lowest detection limits obtained in the LIPS assays to be satisfactory. The LIPS assay for toxoplasmosis could detect antibodies against *T. gondii* in the mouse and human sera with a reasonably high sensitivity.

Conclusion: We developed LIPS assay procedures for toxoplasmosis. It could detect antibody responses in the mouse *T. gondii* infection model. We considered LIPS assay as a promising tool for screening, diagnosis and monitoring toxoplasmosis. Especially, detection of antibodies against BAG1 might be useful for longitudinal sero-prevalence study in suspected high-risk areas based on its elevated serum concentration in the chronic phase.

Keywords: LIPS (luciferase immunoprecipitation systems), NanoLuc (nanoluciferase), *Toxoplasma gondii*, recombinant proteins, TOXM, serodiagnosis

備考　論文要旨は、和文にあっては2，000字程度、英文にあっては1，200語程度