INCREASE OF IMMUNOGLOBULIN A TITER IN MICE INTESTINAL MUCOSA AFTER IMMUNIZATION WITH *TOXOPLASMA GONDII* SOLUBLE PROTEIN WITH AND WITHOUT INTRANASAL CHOLERA TOXIN ADJUVANT

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ABSTRACT

This study was conducted to investigate the difference effect of intranasal immunization of Toxoplasma gondii SPTAg with or without CT adjuvant on IgA intestinal mucosa level. This experimental study was also to find out the interaction effect between combined group of treatment and time of flushing intestinal mucosa collection that influenced IgA intestinal mucosa level. This study was an experimental study designed using factorial 3x4 post test only design. Forty-eight mice were divided into 3 groups: First group was the control group, immunized with PBS (Phosphate Buffer Saline), second group immunized with SPTAg; third group was immunized with SPTAg and CT adjuvant. Immunization was done once at the beginning of the course of this study. Samples of flushing intestinal mucosa, were collected every week up to week four post immunization. The titration of IgA was performed using ELISA method. Data were analyzed using factorial ANOVA test and LSD test. The use of SPTAg without adjuvant increased Ig A significantly, particularly during week 1 (0.669), and decreased during the subsequent week, because degradation of SPTAg by intestinal normal flora. In mice immunized with SPTAg and CT adjuvant showed, that Ig A response was low at week 1 (0.386), but then was followed with significant increase and reached the peak of IgA response at week 3 (0.632). Subsequently, it reduced and became insignificant ($p>0.05$) up to week 4 (0.551). The result showed that immunization with SPTAg and CT adjuvant was able to maintain IgA response of intestinal mucosa longer than that of immunization without CT adjuvant, probably CT adjuvant could reduce the degradation of intestinal flora, and because IgA switching was relatively higher in intestinal mucosa than that in serum. Conclusively, the immunization with SPTAg and CT adjuvant intranasally is able to induce the increase and change of immune response profile better than SPTAg only.

Keywords: *T. gondii SPTAg, CT adjuvant, intranasal, IgA*

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INTRODUCTION

Toxoplasmosis is a disease caused by *Toxoplasma gondii*. It is a zoonosis disease, transmitted from animal to human. The primary source of infection is cats, although a number of fowls and other mammals, including human, may serve as intermediate hosts as well as infection source, so that this disease can disperse worldwide (Soedarto 1995, Gandahusada 2000). The negative effects from toxoplasmosis on health are remarkable. This parasite causes serious problems, particularly for pregnant women, children or immunocompromised patients. The prevalence of congenital toxoplasmosis in several countries is as follows: the Netherlands, 6.5 from 1000 living birth, USA 1.3% and Paris 6-7%. In Indonesia, the prevalence of positive anti-*T. gondii* substance in human is approximately 2%-63%, while in animals is as follows: cats 35%-73%, pigs 11-36%, goats 11-61%, dogs 75% and other cattle less than 10% (Gandahusada 2000).

In human, particularly in pregnant women with toxoplasmosis, *T. gondii* can be transmitted to the fetus. The transmission occurs since *T. gondii* existing in the circulation is able to penetrate placental barrier and spread to entire fetal tissues or organs (Darcy & Santoro 1994, Robert & Janovy 2000). Congenital toxoplasmosis due to fetomaternal transmission can be caused by the presence of primary infection, reinfection or *T. gondii* cysts reactivation in pregnant women (Holliman 1996, Haumont et al 2000). *T. gondii* fetomaternal transmission to the fetus may result in several pathological effects, such as fetal death, stillbirth, and birth with various pathological conditions and retinochoroiditis (Haumont et al 2000; Holliman 1996; Levine 1995; Smith 1999).

Immune response induced in the body due to *T. gondii* infection presents as humoral and cellular immune response, either systemic or local. Local humoral

immune response against toxoplasmosis particularly occurs at the surface of intestinal tract mucosa, since in the natural pathogenesis of toxoplasmosis, the mucosa of intestinal tract is the first entry site of this parasite, so that humoral immune response, mainly performed by secretory IgA (sIgA), is highly important as local defense of intestinal mucosa against *T. gondii* (Brandtzaeg 1994; Underdown & Mestecky 1994).

Current development of toxoplasmosis management is marked by the effort to provide early diagnosis and prevention by developing candidate vaccine for safe and effective immunization. However, until today such vaccine has not been found. A vaccine is considered safe if there is no virulence reactivation of vaccine materials within the body of vaccined individuals or the presence of other negative effects. Vaccine effectiveness is based on its ability in stimulating specific adaptive immune response. Several immunogenes that become the focus of candidate vaccine for toxoplasmosis are tachyzoite surface antigen (SAG), dense granule (GRA) antigen, micronema (MIC) antigen and rhoptri (ROP) antigen from *T. gondii* (Darcy & Santoro 1994; Debard et al 1996; Fischer et al 1996; Ficher et al 1998; Haumont et al 2000; Jacobs et al 1999; McLeod et al 1991; Prigione et al 2000; Velge – Roussel et al 2000; Vercammen et al 2000). Soluble protein can induce anti-SAG1 and -SAG-2 in CBA/J antibody mice. Soluble protein contains many antigens, such as GRA, ROP, MIC or SAG, so that its use may enhance immune response against *Toxoplasma gondii* extensively against various toxoplasma antigenes. Anti-GRA, anti-ROP, anti-MIC, and anti-SAG antibodies are integratively expected to cause inhibition in tachyzoite penetration into the cell (Bourgin et al 1998).

Vaccine effectiveness is also determined by the antigen vaccine application given. Most of the vaccines are administered parenterally in order to stimulate systemic immune response that has been proved to be effective in controlling toxoplasmosis, while *T. gondii* infection occurs naturally through digestive tract and such infection primarily induces local immune response (intestinal mucosa). An effective application to incite local immune response is per oral. However, the digestive tract environment may affect the application of vaccination if it is given per oral. One alternative in the application of vaccination that can induce mucosal immune system is by intranasal administration (Debard et al 1996; Roussel et al 1999). The emergence of systemic or local immune response in intestinal mucosa results from activation and migration of dendrite cells and lymphocyte at induction site (nasal mucosa) to circulation system through posterior cervical lymph nodes and spleen. Some of the lymphocytes also migrate to intestinal mucosal tissue (Bonefant et al 2001). In that target location, the T helper will activate B lymphocyte to produce IgA as a response against antigen (Sminia & Kraal 1999).

The opportunity of antigen degradation and total number needed for induction into the immune system at intranasal activation is relatively lower than that through intramuscular or oral route (Debard et al, 1996). As an example is the study from Barackman et al (1996) shows that the intranasal application of influenza virus hemaglutinin antigen is able to induce immune response stronger than that through intramuscular route. However, the application in such a way also bears a risk of the weakness of immunogeneity of the antigen used, so that a cholera toxin (CT) adjuvant is needed (Debard et al 1996; Douce et al 1997; Rappuoli et al 1999, Velge–Roussel et al 2000).

The use of SPTAg as antigen to induce the formation of specific immunoglobulin has been reported by Darcy and Santoro (1994). The study by Subekti & Arrasyid (2002) also used SPTAg to differentiate CT adjuvant and head labile enterotoxin (LT) in intranasal application. Subcutaneous SPTAg application may provide protection against *T. gondii* (Alexander et al. 1996). Vaccination through subcutaneous route using SPTAg from RH strain *T. gondii* tachyzoit with interleukin (IL) 12 adjuvants also induces protective immune response in BALB/c mice (Yap et al. 1998). In contrast, in intranasal application, SPTAg capability in inducing immune response has not been known, either in single administration without adjuvant or using CT adjuvant. These considerations necessitate the investigation on the difference of immune response induced after immunization with *T. gondii* SPTAg with or without CT as adjuvant. On the other hand, SPTAg evaluation as vaccine material for intranasal application has not disclosed its efficacy in inducing humoral immune response, particularly the IgA.

MATERIALS AND METHODS

This study was an experimental laboratory study using post-test only factorial design (3 x 4). The study was conducted at Interuniversity Central Laboratory (PAU), Biotechnology, Gadjah Mada University School of Medicine, Yogyakarta, from May to August 2004. The subjects of the study were female BALB/c mice aged 6 - 8 weeks with an average weight of 15-20 grams. Samples were taken from infinite population, comprised 48 mice, which were divided into three groups using simple random sampling, i.e. treatment group 1, served as control group, immunized with Phospate Buffered Saline (PBS); treatment group 2, immunized with *T. gondii* SPTAg without CT adjuvant, and treatment

group 3, immunized with *T. gondii* SPTAg with CT adjuvant. Material for immunization was SPTAg isolated from RH strain *T. gondii* tachyzoit obtained from Biotechnology Study Program, Gadjah Mada University, and the adjuvant used was CT adjuvant (Sigma Chem, USA).

Immunization is carried out once at the beginning of the study, intranasally by dripping SPTAg (in PBS) with or without CT adjuvant in mice nostril. The dose used was 1.25 ug/ul (10 ug for each mouse) (Debard et al 1996), while CT adjuvant dose was 1 ug per mouse (Douce et al 1997). Intestinal mucosal fluid of the mice was taken by means of flushing using 2-3 ml PBS containing 2 nM PMSF, 10 ug TPCK, 0,02% NaN3, 5 mM EDTA, penicilline and streptomycin into the intestinal tract that had been cut previously (modification of Yun et al 2000). The flushing fluid was centrifugated 10,000 rpm for 4 minutes, contained within eppendorf and kept in the freezer of -20 degree C until the time of evaluation using ELISA. Flushing was conducted 4 times in 7 days interval. Immunoglobulin A titer measurement was done using indirect ELISA. Data collected from the IgA titer were tabulated and converted into graphs for descriptive presentation. Statistical analysis using factorial anava, LSD test and regression test was subsequently undertaken with significance level of 95% (alpha = 0.05).

RESULTS

The descriptive results of the data are as follows:

Table 1. OD values in each treatment and repetition

Time	Treatment	Repetitions				Mean	SD
		1	2	3	4		
	PBS	0,41	0,286	0,287	0,286	0,317	0,062
Week I	SPTAg	0.706	0.660	0.643	0.670	0.669	0.026
	SPTAg+CT	0.322	0.374	0.485	0.363	0.386	0.0696
	PBS	0.244	0.26	0.286	0.41	0.300	0.075
Week II	SPTAg	0.47	0.578	0.502	0.428	0.494	0.063
	SPTAg+CT	0.621	0.539	0.541	0.567	0.567	0.038
	PBS	0.287	0.391	0.26	0.286	0.306	0.058
Week III	SPTAg	0.422	0.395	0.37	0.429	0.404	0.027
	SPTAg+CT	0.427	0.51	0.747	0.845	0.632	0.196
	PBS	0.391	0.244	0.26	0.287	0.295	0.066
Week IV	SPTAg	0.391	0.457	0.333	0.292	0.368	0.072
	SPTAg+CT	0.7	0.42	0.634	0.45	0.551	0.137

Notes:
PBS = Treatment group receiving PBS
SPTAg = Treatment group receiving SPTAg
SPTAg+CT = Treatment group receiving SPTAg + CT
SD = Standard Deviation

The IgA OD value was positive if the mean OD in each treatment was equal total OD PBS + (2xSD PBS). From the measurement it was found that OD IgA was positive if it was 0.438. The description of IgA immune response based on OD value in each treatment groups (Table 1) is as follows: In treatment group that received PBS as control, the highest OD value in week 1 was 0.317. Treatment with SPTAg showed the average highest OD in week 1 was 0.669, and decreasing in the following weeks. In the group receiving SPTAg+CT, the lowest OD in week 1 was 0.386 and the highest value in week III was 0.632.

Factorial Anava Analysis

This analysis was used to find the difference and interaction between the existing variables.

Table 2. The Results of Factorial Analysis

Factor	F value	P
Time	0.937	0.433
Treatment	30.539	0.000
Treatment Time Interaction	7.191	0.000

Notes:
F = fisher
P=probability

The result of factorial anava analysis in Table 2 showes that the factor of time has no significant difference ($p > 0.05$), so that it can be concluded that the trial in four times showed no significant OD. However, for treatment and interaction between times and treatment showed significant difference ($p > 0.05$). Thus, the treatment in this study revealed significant OD

difference, and the interactions also revealed significant different IgA OD.

Analysis of Least Significant Different (LSD) Test

Table 3. Result of LSD test for observation time

Observation Time Compared		Significance	I Interpretation
Week I	Week II	0.916	NSD
	Week III	0.776	NSD
	Week IV	0.148	NSD
Week II	Week III	0.858	NSD
	Week IV	0.178	NSD
Week III	Week IV	0.241	NSD

NSD = Not significantly different

LSD test for comparison of one observation time and the others revealed no significant difference ($p > 0.05$). However, regression test revealed significant results ($p < 0.05$).

Treatment: SPTAg

Table 4. Result of regression test of time in SPTAg treatment

	DF	Sum of Squares	Mean Square
Regression	2	.21734252	.10867126
Residuals	13	.03199142	.00246088
F=	44.15954	Significance F=	.0000

Table 5. Result of regression test for time in SPTAg+CT treatment

	DF	Sum of Squares	Mean Square
Regression	2	.13155108	.06577554
Residuals	13	.19108986	.01469922
F=	44.47476	Significance F=	.0332

Table 6. Result of LSD test for treatments

Compared treatments		Significance
PBS	SPTAg	0.000
	SPTAg+CT	0.000
SPTAg	SPTAg+CT	0.114

The LSD test to compare between treatments showed that PBS and SPTAg had significant difference ($p < 0.05$), and so did the PBS with SPTAg and CT adjuvant ($p < 0.05$), while SPTAg and SPTAg with CT adjuvant had no significant difference ($p > 0.05$).

Interaction between observation time and treatment

This analysis was carried out to find interaction between observation time and treatment (flushing taking) and treatment.

Table 7. The result of LSD test on the time of observation and treatment

Obsevation time-treatment	Mean	±	SD
Week I-PBS	0.317^{a}	±	0.062
Week II-PBS	0.300^{a}	±	0.075
Week III-PBS	0.306^{a}	±	0.058
Week IV-PBS	0.296^{a}	±	0.006
Week I-SPTAg	0.670^{b}	±	0.027
Week II-SPTAg	0.495^{a}	±	0.063
Week III-SPTAg	0.404^{a}	±	0.027
Week IV-SPTAg	0.368^{a}	±	0.072
Week I-SPTAg + CT	0.386^{a}	±	0.070
Week II-SPTAg + CT	0.567^{b}	±	0.038
Week III-SPTAg + CT	0.632^{b}	±	0.196
Week IV-SPTAg + CT	0.551^{b}	±	0.137

Notes: Coefficient with the same letter indicates insignificance

DISCUSSION

The determination of IgA titer in intestinal mucosal fluid can be based on the optical density (OD) value using ELISA. OD value in ELISA in quantification based on the change of colors from enzyme-substrate changes and read using a spectrophotometer, the ELISA reader. The colour change quantified into OD value had positive correlation with measured immune response. IgA response was regarded as positive if the OD value was higher or similar to the total of average negative control OD added with twice of the standard deviation of negative control OD (Aubert et al. 2000; Paul 1999,).

Based on factorial anava and LSD test (Table 2 and 3), it was apparent that the time of intestinal mucosal fluid taking (flushing) had no influence on IgA immune response, while regression test (Table 4 and 5) showed significant results ($p > 0.05$). In treatment group, interaction between time and treatment showed significant results ($p < 0.05$), (Table 6 and 7). This indicates that treatment by giving SPTAg and SPTAg with CT resulted in significantly different IgA immune response compared to PBS in control group. These results generally confirmed the previous report revealing that the use of intranasal SPTAg demonstrated changes in the pattern and profile of IgA immune response in intestinal mucosal fluid (Subekti & Arrasyid 2002).

Mice immunized intranasally using SPTAg without adjuvant was apparently able to induce IgA response since the first week and steadily decreasing until the end of observation, at the fourth week. This could be observed from the OD value at week I, 0.67, and that at week IV, 0.386 (Table 1). The result was confirmed with statistical analysis, in which the use SPTAg without adjuvant in week I showed significant difference from the use of SPTAg in week II to IV (Table 7). Conversely, in group that used CT adjuvant, IgA response was lower at the first week (0.386), but it was followed with significant increase in the third week (0.632) and the IgA response was maintained until the fourth week (0.551) (Table 1). Statistical analysis revealed that treatment using SPTAg with CT adjuvant also indicated significant results in weekly IgA immune response observed ($p > 0.05$). The significant difference was found between the first week, when the lowest titer was found, and the third week, during which the IgA immune response in intestinal mucosal surface was at peak. The difference in second and fourth week was not significant compared to that in the third week (Table 7). This confirmed the profile of CT-adjuvant-induced IgA response, which tended to decrease slowly as proved by the insignificant OD value in the interval of that week.

IgA response in intestinal mucosa in those receiving intranasal immunization with SPTAg without adjuvant could not last long, even though it could induce IgA faster than that without adjuvant. Similar condiition could also be seen in previous similar studies (Debard et al. 1996; Subekti & Arrasyid 2002). It was suggested that IgA would naturally associate with mucosal later at the surface of intestinal mucosa, while that within the lumen of intestinal tract would be easily degraded, resulting in the fast decrease of concentration. During sample collection, it was found that IgA obtained was commonly the one in mucosal lumen, so that the degradation of IgA in intestinal mucosa might be caused by several normal flora in intestinal tract (Kilian & Russel 1994). However, in immunization using adjuvant, IgA remained within the intestinal lumen. This can be assumed that the bacterium in the adjuvant was able to reduce the IgA degradation by normal flora. The results of some previous studies had showen that a fast IgA reduction could occur in a period of 1 to 2 weeks after immunization without adjuvant (Debard et al. 1996; Subekti & Arrasyid 2002).

The use of CT adjuvant at the first week resulted in temporary suppression, leading to lower immunoglobulin immune response. However, about two and three weeks after the last immunization, there was a significant increase of IgA immune response. This has also been reported by Subekti and Arrasyid (2002) who found that the peak intestinal mucosal IgA titer against SPTAg was reached at fourth and fifth week after booster was carried out at the second week. Debard et al (1996) also reported that the use of CT adjuvant would remarkably induce the increase of intestinal mucosal IgA and reached the peak three weeks thereafter (approximately twenty-five days).

The lower IgA response in group using adjuvant at the early phase occurred since, theoretically, the use of CT adjuvant could result in temporary suppression at the early phase due to the depletion of T lymphocyte (T cells) population, particularly the Th, in induction sites, i.e., the nasal mucosa or nose. The suppression was caused by the increase of intracellular cAMP activity in T cell (Bowman & Clements 2001; Yamamoto et al. 1999,). If the activated T cells are depleted, the total population of activated T cells that will migrate may also decrease. According to Sminia and Kraal (1999) the activated T cells in nasal mucosa would migrate to intestinal mucosa. If the total number of migrated activated T cells reduces, the B cell activation also reduces, resulting in suboptimal response.

Suppression does not last in a longer time and recovery immediately ensues. It occurs since the cytokine that induces IgA switching in intestinal mucosa is produced in a relatively higher number as compared to serum that affects the IgM as well as IgG response. Cytokines, such as IL 5, IL 6 and TGFß, are inductors for plasma cells in differentiating to secrete IgA (Abbas et al. 2000; Mestecky et al. 1999; Kim et al. 1998). It is furthermore stated that IL 5 is produced by Th2 and IL-6 and TGFß are produced by T cells, macrophage as well as epithelial cells commonly found at the surface of intestinal mucosa (Abbas et al. 2000; Mestecky et al. 1999). It implies that the production of IL-6 and TGFß that play an important role in the induction of IgA secretion by plasma cells will be in a relatively higher availability in intestinal mucosa than in the system, so that the increase of IgA response in the mucosa will be immediately detected one week later (two weeks after its use, CT as adjuvant is able to increase IgA response significantly in intestinal mucosa). This was also confirmed in this study, in which from the second to the fourth week IgA response remained detectable with a predisposition of relatively slow decrease. The use of CT adjuvant also maintains IgA specific response against SPTAg, which is steadily higher than that resulting from intranasal immunization without adjuvant in subsequent weeks as that reported by Subekti & Arrasyid (2002).

The result of this study generally demonstrated that the use of CT adjuvant, despite intranasal application, was still able to induce increase and change in IgA immune response profile better than that obtained in intranasal

immunization using SPTAg without adjuvant. Other studies reported by Debard et al. (1996) revealed similar results in the induction of IgA immune response, although the antigen used was different. IgA is the primary immunoglobulin that plays a major role in adaptive immune response in intestinal mucosa. IgA can interact synergistically and additively with mucosal layers of the intestine to neutralize infectious microorganisms entering the body through alimentary tract. The presence of IgA immune response increase and its ability to maintain it in a longer time can be expected to provide early protection that may reduce and prevent as well as eliminate the possibility or microorganism entrance into the circulatory system.

CONCLUSIONS

Conclusively, the immunzation using SPTAg without CT adjuvant and SPTAg with CT adjuvant applied intranasally may enhance IgA immune response as compared to control (PBS). Time of observation has influence on intestinal mucosal IgA due to the administration of SPTAg and SPTAg with CT adjuvant applied intranasally. There is also an interaction between time of observation and treatment with the increase of intestinal mucosa IgA. It is suggested to conduct further studies by using different type of *T. gondii* protein. Other variables, such as IL-4, IL-5, IL-6, IL-13, IL-12, IFN-gamma as well as TGFß, should be involved to disclose more detail the difference in the change of immune response profile induced by CT adjuvant.

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