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Intranasal Immunization with *Toxoplasma gondii* SAG1 Induces Protective Cells into Both NALT and GALT Compartments

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Intranasal (i.n.) immunization with the SAG1 protein of *Toxoplasma gondii* plus cholera toxin (CT) provides protective immunity. The aim of this study was to analyze the cellular activation of several mucosal compartments after i.n. immunization. Cervical and mesenteric lymph node (CLN and MLN, respectively) lymphoid cell and intraepithelial lymphocyte (IEL) passive transfer experiments were performed with CBA/J mice immunized i.n. with SAG1 plus CT. CLN and MLN cells and IEL isolated 42 days after immunization conferred protective immunity on naive recipient mice challenged with strain 76K *T. gondii*, as assessed by the reduction in the number of brain cysts. There were proliferative specific responses in nose-associated lymphoid tissue and the CLN and MLN cells from mice immunized with SAG1 plus CT, but no cytokine was detectable. Thus, protective immunity is associated with a specific cellular response in the nasal and mesenteric compartments after i.n. immunization.

Infection with *Toxoplasma gondii* confers on the host lifelong protective immunity against reinfection. This suggests that prevention of toxoplasmosis is a realistic goal (22).

Studies showing the importance of systemic cell-mediated immunity in protection against toxoplasmosis are unequivocal (9, 11). Infection with *T. gondii* generally occurs via the oral route and triggers a cellular response in the gut (5, 6). Anti-*T. gondii* cellular mucosal immunity is strongly mediated by lymphocytes, mainly intraepithelial lymphocytes (IEL) (2, 19).

Some attempts to generate protective immunity against toxoplasmosis have used the peroral route (1). Since all mucosal surfaces have the same mucosal immune system, the intranasal (i.n.) route is a possible alternative for immunization (18). In a previous study, the use of SAG1, the major surface antigen of *T. gondii*, plus cholera toxin (CT) for i.n. immunization provided potent protection, reducing the cyst burden by 80% (8). Thus, protective immunity can be stimulated by i.n. immunization of the intestine, where the pathogen enters the host. This study was carried out to investigate the mucosal immunity induced by i.n. immunization with SAG1 plus CT.

Parasites. Cysts of the 76K *T. gondii* strain were obtained from the brains of orally infected CBA/J mice (Janvier, St Genest/Lisle, France). Tachyzoites of the virulent strain RH of *T. gondii* were harvested from peritoneal fluid of Swiss OF1 mice infected intraperitoneally and were used to prepare the sonicated antigen (toxoplasmic extract) (6).

Mouse immunizations. Eight CBA/J mice were immunized i.n. with 20 μg of *T. gondii* SAG1 plus 0.5 μg of CT (Sigma) at 10 μl per nostril under light ether anesthesia and boosted 4 weeks later in the same way. SAG1 was purified using an anti-SAG1 monoclonal antibody (MAb) affinity column (16), and the purity of the protein was demonstrated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis analysis in a PHAST system (Pharmacia), followed by silver staining of the gel (16).

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Adoptive transfer of cells. Immunized CBA/J mice were killed on day 15 after the boost, and cell suspensions were prepared from cervical and mesenteric lymph nodes (CLN and MLN, respectively) and the small intestine. IEL were prepared as previously described (4). Cells (5×10^5) were analyzed with an anti-CD8β MAb (53-5.8 clone; PharMingen) and an anti-Thy 1.2 MAb (53-2.1 clone; PharMingen) on a FACScan (Becton Dickinson, Mountain View, Calif.). Around 3×10^7 and 2×10^6 lymph node cells and IEL, respectively, were intravenously injected into CBA/J recipient mice (six per group).

The day after the adoptive transfer, recipient mice were infected orally with 80 cysts of *T. gondii* strain 76K. Mice that received IEL were challenged 4 days after cell transfer. Intracerebral cysts were counted 1 month later. Brains were removed and homogenized in 5 ml of phosphate-buffered saline. The average number of cysts per brain was determined by examining eight samples of each homogenate (10 μl each). Results were collected from at least six mice per group and were expressed as the average ± the standard deviation for each group. The analysis of variance (ANOVA) test was used to determine the statistical significance level of differences.

Proliferative T-cell response. Single-cell suspensions were prepared 15 days after the boost by purifying the cells from CLN, MLN, and nasal tissue (NALT) from four immunized mice. To isolate NALT cells (27), mice were killed by ether asphyxia, the lower jaws were removed, and the palates were cut parallel to the septum and removed. The NALT from each side of the septum was gently removed with fine dissection forceps. Cell suspensions were prepared by passing the tissue suspended in RPMI 1640 medium through nylon net (Blutex; Tripett et Renaud) using a syringe. All cells (5×10^5) were incubated for 5 days with different antigen (toxoplasmic extract) concentrations in RPMI 1640 medium (Gibco) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg of gentamicin per ml, 5×10^{-5} M 2β-mercaptoethanol, and 5% fetal calf serum. Proliferative responses were assessed over 18 h by measuring [^{3}H]thymidine (18.5 kBq per well [Dositek]) incorporation. Incorporated radioactivity was measured in a liquid scintillation counter (LKB) following filtration on fiberglass filters. Results were expressed as the mean of triplicates ± the standard deviation.

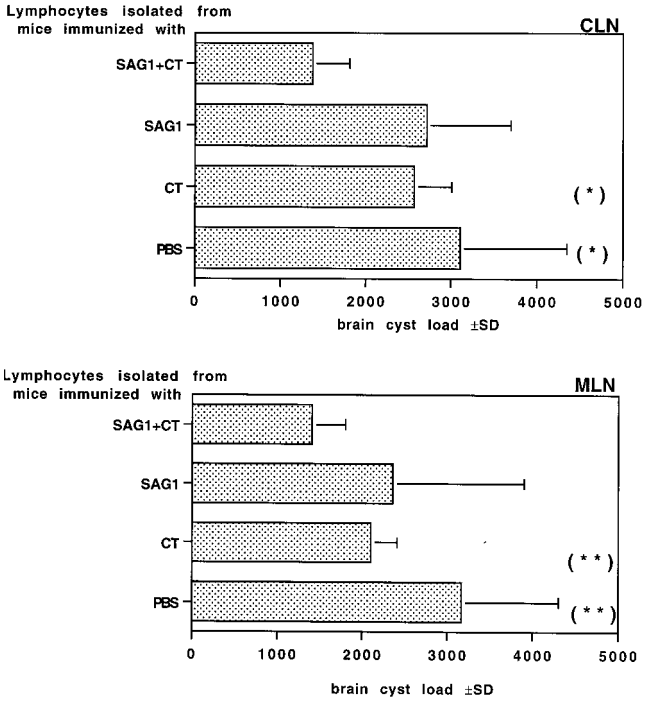

FIG. 1. Adoptive transfer of CLN and MLN primed with SAG1 plus CT to CBA/J mice. For MLN and CLN adoptive transfer, six naive mice were given around 3×10^7 cells from donor mice. They were challenged by the oral route with 80 cysts of strain 76K *T. gondii* 1 day after the transfer. Protection was assessed by counting the cysts in the brain 1 month later. Statistical significance was calculated by the ANOVA test as indicated by asterisks. For the SAG1-plus-CT group compared to the phosphate-buffered saline (PBS) or CT group: **, $P < 0.0003$; *, $P < 0.01$. The results are representative of two independent experiments.

CLN and MLN cells and IEL of i.n. immunized mice transfer protective immunity. CBA/J mice are resistant to acute *T. gondii* infection but develop a large number of brain cysts. The protection conferred by adoptive transfer of lymphocytes collected on day 42 from organs of i.n. immunized mice was evaluated. One month after infection, CBA/J mice given MLN or CLN cells from mice immunized with SAG1 plus CT exhibited significantly fewer cysts (50 and 60% reduction; ANOVA test, $P < 0.0003$ and $P < 0.01$, respectively) than naive mice or mice given lymph node cells stimulated with CT alone in vivo (Fig. 1). Thus, lymphocytes from CLN and MLN were stimulated by i.n. immunization with SAG1 plus CT and participated in the protection of recipient mice against *T. gondii* challenge. IEL from mice immunized with SAG1 plus CT were also transferred to recipient mice (Fig. 2). One month after the challenge, the protection conferred by IEL was similar to that obtained with MLN and CLN cells (about 50%) compared to controls.

Proliferative response of NALT, CLN, and MLN cells. In order to study activation of the mucosal compartments further, proliferative T-cell responses of NALT, CLN, and MLN were tested. Table 1 shows that a stronger specific proliferative response of NALT cells from the group immunized with SAG1 plus CT was observed when they were incubated with toxoplasmic extract compared to those from the group immunized with CT. Also, cells from the MLN and CLN of these mice also produced a cellular response to toxoplasmic extract incubation (Table 1). Thus, i.n. immunization seemed to induce a specific T-cell response in NALT, CLN, and MLN.

The cells from mice immunized with SAG1 plus CT were purified from NALT, CLN, and MLN and stimulated with

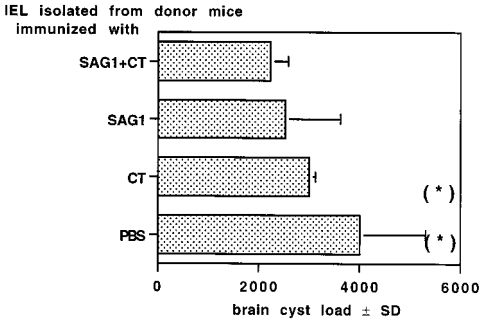

FIG. 2. Adoptive transfer of SAG1-plus-CT-primed IEL to CBA/J mice. Six naive CBA/J mice were given 2×10^6 primed or unprimed IEL from donors and challenged by the oral route with 80 cysts of strain 76K *T. gondii* 4 days after the transfer. These results are representative of two independent experiments. **, $P < 0.01$. PBS, phosphate-buffered saline.

toxoplasmic extract to test their capacity to produce interleukin-10 or gamma interferon. A small amount of gamma interferon (20 to 67 pg/ml) was produced, but no interleukin-10 could be detected (data not shown).

SAG1-primed IEL have mainly the CD8αβ$^+$ phenotype. IEL are mainly CD8α$^+$ cells, which comprise CD8αα$^+$ and CD8αβ$^+$ cells (12, 15). A fluorescence-activated cell sorter analysis was performed to explore the change within the IEL population after i.n. immunization (Fig. 3). There was an increase in the Thy-1.2$^+$ and CD8β$^+$ IEL subsets on day 11 following the boost in the groups immunized with SAG1 plus CT and CT. The phenotypic change that occurs within the IEL population after SAG1 immunization might indicate that intestinal IEL are sensitized by immunization by the i.n. route.

Although NALT contains more naive than memory T helper cells (28), there is specific local proliferation after i.n. immunization. Moreover, proliferation of CLN may indicate that this specific response was extended to the systemic compartment (20, 28). MLNs also showed specific proliferation. The MLN response might indicate that the stimulation was extended to the common mucosal compartment, and thus, NALT appears to be the origin of a specific mucosal response (7, 8, 21, 24).

IEL are important in the gut mucosal immune system, as they are the first functional barrier to toxoplasmosis, having cytotoxic activity and secreting cytokines (2, 4, 19). Our previous studies demonstrated that the CD8αβ$^+$ subset of IEL from

TABLE 1. Proliferation of lymphocytes from the NALT, CLN, and MLN of mice immunized i.n.[a]

Cell	Immunization group	Δcpm[b] of toxoplasmic extract (10 μg/ml)
NALT	SAG1 + CT	12,223 ± 320[c]
	CT	0
CLN	SAG1 + CT	3847 ± 481[d]
	CT	77 ± 65
MLN	SAG1 + CT	10,013 ± 1276[e]
	CT	797 ± 261

[a] Proliferative responses of cells from NALT, CLN, and MLN of mice immunized with SAG1 plus CT cultured in vitro with *T. gondii* total extract for 4 days were measured. Cells were taken from organs on day 42 after immunization, and proliferation was evaluated by measuring [^{3}H]thymidine incorporation. Welsh's test of Instat gave the levels of statistical significance.

[b] Δcpm was calculated as the counts per minute obtained in the presence of stimulating antigen minus the counts per minute in medium alone.

[c] $P = 0.0002$.

[d] $P = 0.0055$.

[e] $P = 0.0066$.

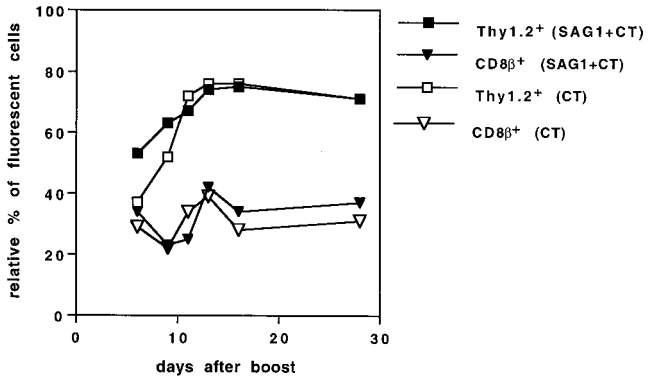

FIG. 3. Phenotypic analysis of gut IEL subsets from CBA/J mice after immunization with SAG1 plus CT. Lymphocyte suspensions were prepared from four mice, and the relative percentages of Thy1.2$^+$ or CD8β^+ fluorescent IEL were determined by cytofluorimetric analysis of 5×10^5 cells.

mice infected with *T. gondii* 11 days before can protect naive mice from a *T. gondii* challenge (2, 19). The increase in the number of Thy 1.2$^+$ CD8β^+ IEL following SAG1 immunization indicates IEL activation. As expected, IEL primed with SAG1 plus TC conferred less protective immunity on recipient mice than did IEL from infected mice (2). Nevertheless, the capacity of primed IEL to protect could be crucial because of their gut location and furthermore because they might be functional early following infection (3).

Since MLN are the first lymphatic organization draining the gut, activation of the lymphocytes at this site might account for protection. Like IEL, MLN lymphocytes might migrate to the gut after their transfer and thus help in blocking parasite invasion (3, 13, 17, 25). Immunization by the i.n. route induces a subset of T helper cells in the CLN and MLN that are implicated in protection (50 and 60%, respectively). Passive transfer of SAG1-primed splenocytes conferred only 30% cyst reduction, whereas 80% cyst reduction was obtained by i.n. immunization with SAG1 plus CT (8). Protective status conferred by i.n. immunization with SAG1 plus CT might be due to the combination of the mucosal immunity represented by the primed IEL and the MLN cells, while the systemic immunity was represented by the spleen (8).

Many studies have used the i.n. route for immunization with potential vaccines with or without CT (10, 14, 23, 26). Vaccinations with aeropharyngeal pathogens have been shown to be successful. They were shown to trigger both mucosal and systemic T- and B-cell responses (20, 28) and thus can be used to target pathogens that invade far from the immunization site, such as the gut or vagina (7, 8, 21, 24). Indeed *T. gondii* naturally invades the intestine of its host and can be partially controlled by i.n. immunization with the protein SAG1 plus CT (8). Our work has demonstrated that this route of immunization induces mucosal cell populations able to participate in protective immunity. Thus, mucosal immunization, particularly via the i.n. route, has considerable potential for triggering immunity in all mucosal and systemic compartments.

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