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Immune Responses in Experimental Toxoplasmosis in Mice Treated with Carrageenan

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Introduction

The mechanisms of protective immunity against *Toxoplasma gondii* (Tp) infection have not been analyzed in detail. Most of evidence accumulated up to now indicates that cell-mediated immunity is the major defense mechanism of the host against this infection (Anderson and Remington, 1974; Sethi *et al.*, 1975; Frenkel and Taylor, 1982). Although antibodies do not appear to play a critical role in the protection against Tp infection, the *in vitro* treatment with heat-inactivated Tp-immune serum before infection empowers the macrophages to inhibit or kill the organisms (Anderson and Remington, 1974; Sethi *et al.*, 1975; Anderson *et al.*, 1976). Antibody plays a crucial role in a balanced host-parasite relationship in Tp-infections *in vivo* (Krahenbuhl *et al.*, 1972; Hafizi and Modabber, 1978). On the other hand, according to Cox (1982), the macrophage activation reinforced by antibody is reported to be the major immune mechanism.

There are many reports evaluating the T cell function in murine models of Tp infection. The growth inhibitory factor of Tp (Toxo-GIF), an immune mediator, is released from sensitized T cells with other similar substances collectively known as lymphokines (LKs) (Shirahata *et al.*, 1977), and inhibits the multiplication of this parasite in mouse macrophages, kidney cells and embryonic fibroblasts (Chinchilla and Frenkel, 1978; Matsumoto *et al.*, 1981). Because *Toxoplasma* grows primarily in somatic cells, Toxo-GIF may be an important factor for the protection. Thus, in nature, it is likely that the combination of antibodies and the lymphocyte-macrophage system affects on the survival and severity of infection in the acute and chronic stage.

Carrageenan (CGN), a sulfated polygalactose of high molecular weight extracted from seaweeds, has been reported as toxic to macrophages but not to lymphocytes (Thomson *et* al., 1976; Rumjanek *et al.*, 1977; Ishizaka *et al.*, 1977), and has been used widely for *in vivo* studies as an immunosuppressive material. Antibody responses are noticeably suppressed by pretreatment with CGN in experimental murine models (Ishizaka *et al.*, 1977; Oka *et al.*, 1981).

In this experiment, mice were treated with CGN before immunization with Tp cell homogenate or before challenge with bradyzoite, or both. The resistance, antibody responses and Toxo-GIF activities in mice were observed to know the role of humoral antibody, phagocytic macrophages and Toxo-GIF in LKs in establishing the protective immunity against Tp bradyzoite (Beverley strain). The results suggest that humoral antibody may not play the crucial role at the initial stage of Tp infection, and even phagocytic macrophages

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may be only little effective on the protection. Therefore, it seems that a certain cooperative activity with Toxo-GIF and somatic cells by way of effector cells may be important in protective immunity against acute toxoplasmosis.

Materials and Methods

Mice: Female ddY mice of a closed colony were obtained from Tokushima Experimental Animal Laboratory, Tokushima, Japan. The age and strain of all immune mice were matched with those of nonimmune mice throughout the experiment.

Parasites: A relatively low virulent Beverley strain was maintained to keep chronic infections by passage of Tp brain cysts in mice. Bradyzoites were prepared by the modified method of Nakabayashi and Motomura (1968) by Ito *et al.* (1976). A virulent RH strain was maintained by serial passages in mice. The tachyzoites which were obtained from peritoneal exudates of mice infected with RH strain organisms 3 to 4 days previously were passed through a CF-11 column and then centrifuged to eliminate host cells. The filtrate was used for an antigen.

Preparation of antigen: The separated parasites were disrupted in a few milliliters of PBS by a 3-time freeze-thawing. The disrupted-cell suspension was repeatedly homogenized with a teflon homogenizer and used as immunogen.

Immunization and challenge: Mice were immunized with Tp cell homogenate (200 μg-protein per mouse), and challenged with 5×10^3 bradyzoites 4 weeks after immunization. Twenty days after the challenge, the immune status of the mice was evaluated by observing the number of mice that survived and the mean survival days of the dead mice.

Treatment of mice with CGN: CGN (Sigma Chemical Co., USA) was dissolved in physiological saline at a concentration of 4 mg per ml and sterilized by autoclaving at 120°C for 15 min. Mice were injected intraperitoneally four times with 0.25 ml of CGN solution on days -7, -5, -3 and -1 (day 0=immunization or challenge, or both).

Antibody measurement: Antibody titer against Tp was measured by latex agglutination using a commercial kit (Eiken Chemical Co., Tokyo). Antibody titers are expressed as $\log_2$ of the highest serum dilution showing complete agglutination.

Preparation of spleen cells and LKs: LKs were prepared as described previously (Nagasawa *et al.*, 1980). Spleen cells separated from Tp-immune, Tp-infected or normal mice, were washed twice with heparinized HBSS (10 units heparin/ml). The resultant cells were resuspended in medium Tc-199 containing 10 % heat-inactivated fetal calf serum and antibiotics (100 units penicillin and 100 μg streptomycin/ml of the medium) to a concentration of 1×10^7 cells/ml. The cultures were incubated with the optimal concentration of 50 μg-protein/ml of Tp lysate antigen (TLA) (Sethi *et al.*, 1975) at 37°C for 48 hr in a humidified atmosphere containing 5 % CO_2, followed by 1,700 $\times$g centrifugation at 4°C for 30 min. The supernatants, hereafter referred to as LKs, were stored at -80 °C until use.

Assessment of Toxo-GIF activity: The adherent cells which were derived from mouse-peritoneal exudates induced by 0.2 % glycogen were used as macrophages. The cells were cultured in a multidish tray (FB-15-24, Limbro Chemical Co., USA) with a round coverslip,and used as macrophage monolayers for the assay of Toxo-GIF activity. The monolayers were infected by replacing the medium with 1 ml of Tc-199 containing 1×10^5 tachyzoites. The infected cultures were incubated at 37°C in a CO_2 air atmosphere for 1 hr, and then washed thoroughly to remove extracellular parasites. Thereafter, LKs were added and the monolayers were reincubated at 37°C. Fourty eight hours after inoculation, the cultures were stained with May-Grünwald Giemsa stain. The Toxo-GIF activity was examined under the light microscope to count the number of uninfected macrohages among 500 macrophages on each coverslip. To convert the Toxo-GIF activity assessed *in vitro* into *in vivo*, the toxoplasmacidal activity of each LKs *in vivo* was calculated by the following formula:

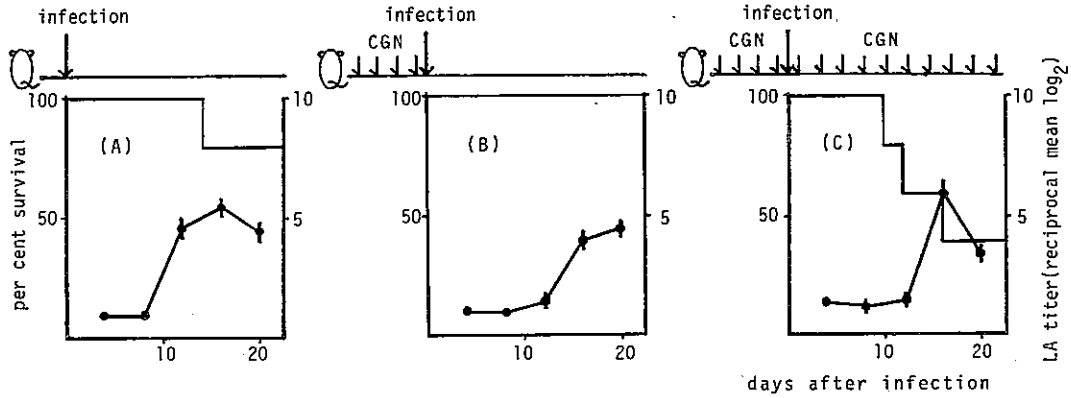

Fig. 1 Effect of treatment with carrageenan on antibody responses and resistance to *Toxoplasma* bradyzoite infection in nonimmune mice. Mice were infected with 50 bradyzoites. (A) : untreatment with carrageenan, (B) : treatment with carrageenan before infection, (C) : treatment with carrageenan before and after infection. Vertical bars represent standard errors.

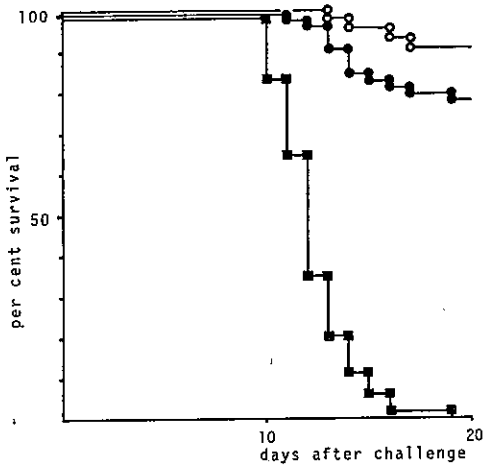

Fig. 2 Resistance to the challenge with bradyzoite in mice pretreated with carrageenan before immunization with *Toxoplasma* cell homogenate.
○—○ : carrageenan-treated Tp-immune mice
●—● : carrageenan-untreated Tp-immune mice
■—■ : carrageenan-untreated nonimmune mice

$$\frac{\text{per cent Tp-uninfected macrophage in I-LKs}}{\text{per cent Tp-uninfected macrophage in N-LKs}} \times \frac{\text{total number of spleen cells of immune mouse}}{\text{total number of spleen cells of normal mouse}}$$

I-LKs : supernatant of immune spleen cells cultured with TLA

N-LKs : supernatant of normal spleen cells cultured with TLA

Results

Effect of treatment with CGN on antibody response and resistance to infection in nonimmune mice : To examine the effect of treatment with CGN on antibody responses and resistance, mice were infected with a sublethal dose of 50 bradyzoites as a preparatory experiment. Fig. 1 shows that the antibody titers of either CGN-treated or untreated mice increased on days 12 and 8, respectively, after infection. No effect of the treatment before infection on the resistance was observed in mice, but the survival rate of these mice was lowered when mice were continuously treated with CGN after infection.

Resistance to the challenge in mice treated with CGN before immunization : All of the nonimmune mice infected with 5×10^3 of bradyzoites died within 20 days after infection, and mean survival days of the dead mice was 12.4±0.3. A strong resistance to the challenge was inducible by immunization with Tp cell homogenate irrespective of CGN treatment. Fig. 2 shows that per cent survival in the CGN-treated immune and untreated immune mice was 88.9 (32/36) and 77.6 (45/58), respectively. Mean survival days of the dead mice of both groups were prolonged significantly (15.0±0.9 and 14.2±0.6) compared with those of nonimmune mice. All of the survived mice showed acute symptoms on

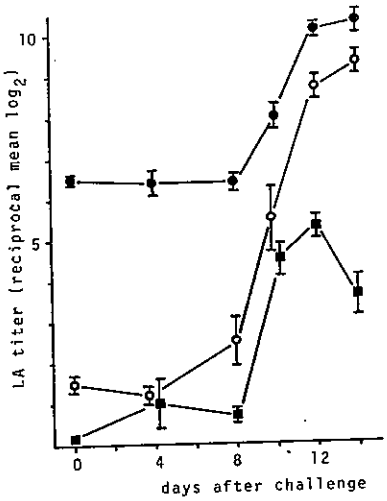

Fig. 3 Antibody response after challenge with bradyzoite in mice pretreated with carrageenan before immunization with *Toxoplasma* cell homogenate. O—O : carrageenan-treated Tp-immune mice, ●—● : carrageenan-untreated Tp-immune mice, ■—■ : carrageenan-untreated nonimmune mice. Vertical bars represent standard errors.

about 8 days after the challenge, and Tp cysts were demonstrable in the brain 2 or 3 months after infection.

Antibody responses to the challenge in mice treated with CGN before immunization: A primary antibody response in mice treated with CGN before immunization was suppressed completely. The antibody titer was less than 1 : 4. After challenge with parasites, however, the antibody titers of them were kept at a same level until 8 days and then increased rapidly reaching the similar level to those of untreated immune mice on day 14 (Fig. 3). In both untreated immune mice and nonimmune mice, increase of the titer was also observed on and after day 8.

Toxo-GIF activity of LKs after challenge in mice treated with CGN before immunization: LKs obtained from spleen cells of immune or nonimmune mice and cultured with TLA were prepared on days 0, 2, 4, 6 and 8 (day 0=challenge), and their toxoplasmacidal activities to normal mouse peritoneal macrophages infected with tachyzoite were examined. LKs obtained from nonimmune mouse on days 6 and 8 showed a slight Toxo-

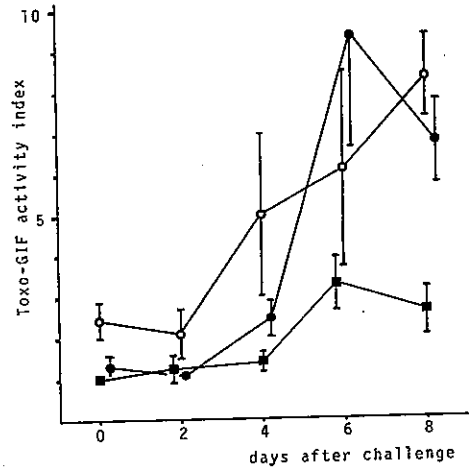

Fig. 4 Toxo-GIF activity of lymphokines after challenge with bradyzoite in mice pretreated with carrageenan before immunization with *Toxoplasma* cell homogenate. O—O : carrageenan-treated Tp-immune mice, ●—● : carrageenan-untreated Tp-immune mice, ■—■ : carrageenan-untreated nonimmune mice. Vertical bars represent standard errors.

GIF activity. LKs obtained from both CGN-treated immune and untreated immune mice on day 4 or 6 showed increasing inhibitory effects on Tp multiplication in macrophages, and those obtained on day 8 showed a remarkable Toxo-GIF activity (Fig. 4). In immune mice, the high Toxo-GIF activity of LKs cultured with and without TLA maintained till days 21 and 14, respectively.

Effect of treatment with CGN before challenge on immune responses: To elucidate the role of macrophages in elimination of parasites in the early stage after challenge, 4 weeks after immunization, CGN-treated immune and untreated immune mice were further given CGN four times before the challenge. Resistance to the challenge, antibody responses and Toxo-GIF activities are shown in Figs. 5, 6 and 7, respectively. None of nonimmune mice could overcome the challenge and mean survival days of the dead mice were not affected by treatment with CGN before the challenge. On the contrary, per cent survival of CGN-treated immune mice was significantly ($p<0.05$) lowered from 88.9(Fig. 2) to 66.7(14/21)by further treatment

with CGN before challenge with parasites. The value of untreated immune mice was also lowered from 77.6 to 70.0 (14/20) by the treatment. Mean survival days of the dead mice in both groups were similar to those in the CGN-untreated controls before the challenge (Fig. 5).

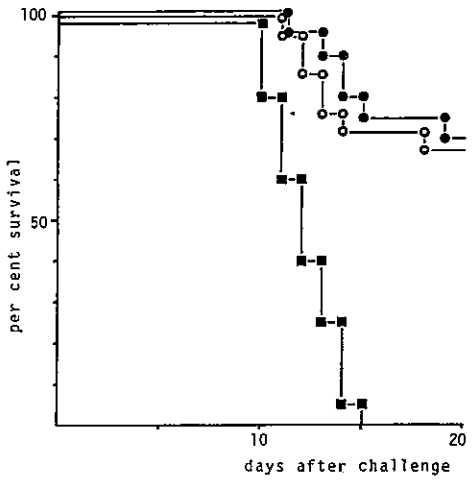

Fig. 5 Resistance to the challenge with bradyzoite in mice pretreated with carrageenan before challenge. O—O : Tp-immune mice treated with carrageenan before immunization and challenge, ●—● : Tp-immune mice treated with carrageenan before challenge, ■—■ : nonimmune mice treated with carrageenan before challenge.

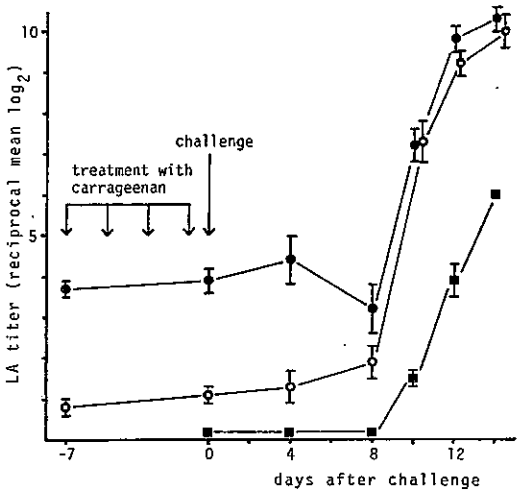

Fig. 6 Antibody response after challenge with bradyzoite in mice pretreated with carrageenan before challenge. O—O : Tp-immune mice treated with carrageenan before immunization and challenge, ●—● : Tp-immune mice treated with carrageenan before challenge, ■—■ : nonimmune mice treated with carrageenan before challenge. Vertical bars represent standard errors.

The treatment with CGN before the challenge did not exert much effect on the secondary antibody responses in mice after the challenge. Increases in the titer were observed on and after day 8 of the challenge as untreated controls (Fig. 6).

The rise of Toxo-GIF activities was remarkably delayed by the treatment with CGN before the challenge compared with an appearance period of the activity in untreated controls. The increased activities in CGN-treated and untreated immune mice were observed on day 8, although these activities remained at low levels until 6 days after the challenge (Fig. 7).

Discussion

This study was designed to know the role of humoral antibody, phagocytic macrophages

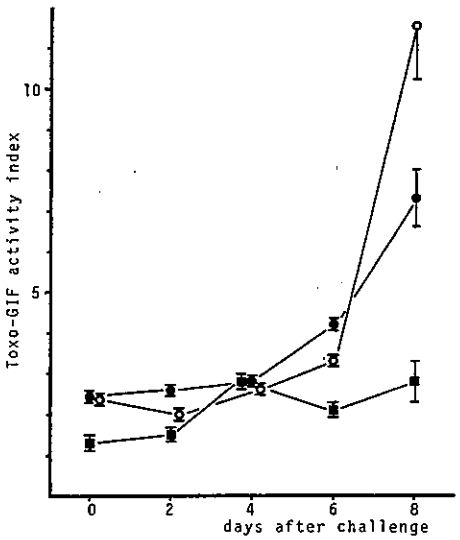

Fig. 7 Toxo-GIF activity of lymphokines after challenge with bradyzoite in mice pretreated with carrageenan before challenge. O—O : Tp-immune mice treated with carrageenan before immunization and challenge, ●—● : Tp-immune mice treated with carrageenan before challenge, ■—■ : nonimmune mice treated with carrageenan before challenge. Vertical bars represent standard errors.

and Toxo-GIF in LKs under the acute toxoplasmosis in mice treated with CGN before immunization with Tp-cell homogenate or before challenge with bradyzoite, or both.

CGN is regarded as a functional inhibitor of phagocytic macrophages but not a functional interferer of T and B cells. Ishizaka *et al.* (1977) reported that pretreatment of mice with CGN induced a noticeable suppression on antibody responses to T cell dependent antigens but did not inhibit T cell independent antigens. Rumjanek *et al.* (1977) described that nonadherent spleen cells from CGN-treated mice responded to phytohemagglutinin as well as cells from nontreated mice. In this study, primary antibody responses in mice to Tp-cell homogenate were markedly suppressed by treatment with CGN before immunization. While no suppression on the secondary antibody responses after the challenge was observed in mice treated with CGN before first immunization as well as before challenge. These results may suggest that most of components of Tp-cell homogenate are T cell stimulative antigens. Oka *et al.* (1981) reported that primary antibody responses to *Trypanosoma gambiense* cell homogenate were suppressed in mice treated with CGN before the first immunization, but reverse was case with response to the second immunization. And they suggested that the memory pool of T cell is expansible by treatment with CGN. The result in this study may support their suggestion.

Resistance to the challenge in the antibody-suppressed mice was rather increased than that in untreated immune mice having high level of antibody titer. This suggests strongly that the level of antibody titer at the time of Tp infection may seldom reflect the protective rate after the challenge with Tp. Fig. 1 proves that nonimmune mice treated with CGN before infection with 50 bradyzoites shows no effect on resistance, but the protection of these mice is lowered by continuous treatment with CGN at the time when antibody titers are high. And antibody-suppressed mice treated with CGN before immunization shows the same level of antibody responses as controls 14 days after challenge (Fig. 3). Hafizi and Modabber (1978) and Frenkel and Taylor (1982) reported that antibody played a significant role to give resistance to Tp in mice treated with cyclophosphamide and sulfadiazine, respectively. From all results, it seems that humoral antibody may not play a decisive role by itself in elimination of Tp at the time of infection or in the early stage of the infection, but play an important role in eliminating Tp in acute infection or in controlling long-term toxoplasmosis.

In nonimmune mice treated with CGN before infection with sublethal dose of parasites, the difference of survival rate between CGN-treated mice and untreated control was not recognized. Per cent survival of CGN-treated immune mice was significantly lowered by treatment with CGN before challenge as compared with that of untreated control. According to Ishizaka *et al.* (1977), the kinetic studies of a CGN action on antibody response showed that its effect may continue for at least 5 days. These findings suggest that phagocytosis by normal macrophages may not play an important role in elimination of Tp, and even activated macrophages in immune mice might give little effect on elimination of invader at the time of infection. The studies of Swartzberg *et al.* (1975) and Hof *et al.* (1976) supported these findings. They suggest that activated macrophages play a little or no role in resistance to Tp *in vivo*.

Tp multiplies intracellularly and spreads cell-to-cell. The anti-Tp antibody does not have a direct effect when the organisms are already in cells. Therefore, cellular mediator such as Toxo-GIF in LKs may be a very important factor on protection. Toxo-GIF is released from sensitized T cells (Shirahata *et al.*, 1977) and inhibits the multiplication of intracellular parasites not only in macrophages but also in other somatic cells (Chinchilla and Frenkel, 1978; Matsumoto *et al.*, 1981). Shirahata and Shimizu (1980) reported that Toxo-GIF, interferon-γ-like substance, can be used as an indicator of T-cell mediated immunity. In this study, to regard the Toxo-GIF activity as an indicator of cel-

lular immunity *in vivo*, the activity was evaluated by a formula. This formula might be justified from result that the level of Toxo-GIF activity should be dose-dependent on the number of spleen cells incubated with TLA. Fig. 4 shows that the Toxo-GIF activities in CGN-treated immune mice and untreated immune mice increase on and after days 4 and 6, respectively. On the contrary, Fig. 7 shows that the rise of Toxo-GIF activities is remarkably delayed by the treatment with CGN before challenge. The per cent survival of CGN-treated immune mice was significantly lowered by the treatment with CGN before challenge as compared with that of untreated control. Furthermore, the high activity of Toxo-GIF in LKs cultured with and without TLA in immune mice continued till days 21 and 14, respectively, after the challenge. The mice showed acute symptoms from about 8 to 14 days after the challenge. Therefore, it appears that the Toxo-GIF activity may play an important role in the protection against Tp infection.

In conclusion, it is suggested on the mechanisms of protective immunity against toxoplasmosis (1) that phagocytosis of nonimmune macrophages gives only little effect, (2) that the cellular factors including LKs take part in the activation of immune effector cells and other somatic cells, and (3) that the subsequent interaction between cellular mediators and humoral antibody plays important roles in conferring resistance and controlling toxoplasmosis.

Summary

The relative roles of humoral immunity and cellular immunity in controlling *Toxoplasma* infection have not been clearly defined. The work reported here is designed to elucidate the mechanisms of protective immunity in mice with toxoplasmosis. Mice were treated with carrageenan (CGN) before immunization with 200 μg protein of Tp cell homogenate or before the challenge with 5×10^3 bradyzoites of Beverley strain, or both. All nonimmune mice died within 20 days after the challenge, and about 80 % of the immune mice survived. Treatment with CGN before immunization completely suppressed the primary antibody responses, while no suppression on the secondary antibody responses after challenges was observed in mice treated with CGN before the first immunization as well as before the challenge. Survival rate of these antibody-suppressed mice was similar to that of untreated immune mice. Treatment with CGN before the challenge resulted in a decreased resistance and the delayed appearance of toxoplasmacidal activity in lymphokines in CGN-treated immune and untreated immune mice. Per cent survival of nonimmune mice was lowered by continuous treatment with CGN at the time when antibody titer was high. These findings suggest that the initial defensive mechanisms by humoral antibodies or activated macrophages give only little effect on the protection, but both cellular mediators which activate somatic cells and subsequent interactions between these factors and humoral antibodies play an important role in the protection.

Acknowledgements

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Carrageenan 処置マウスの実験的トキソプラズマ症に対する免疫応答

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Toxoplasma gondii (Tp) 感染に対する防御免疫機構を解明する目的で，マクロファージ障害性物質である carrageenan (CGN) を用いて実験を行つた．防御抗原性の高い Tp cell homogenate による免疫前に CGN を投与したマウスは，一次抗体産生が顕著に抑制されるが，免疫4週間後の bradyzoite (Beverley 株，5×10^3 個) 攻撃に対し，CGN 無処置の免疫マウスと同様に高い生存率を示した．マウス脾細胞由来のリンホカイン中の1因子で抗 Tp 作用を持つ Tp growth inhibitory factor (Toxo-GIF) は CGN 処置の免疫マウスで攻撃4日目から，CGN 無処置の免疫マウスで6日目からその活性が認められ，攻撃後8日目の値はともに非免疫マウスのそれの約2.7倍であつた．両免疫マウスの攻撃後の二次抗体応答は8日目以降に認められた．非免疫マウスに bradyzoite (50個) を感染し，感染前から抗体産生以後まで CGN 処置を続けると，無処置の対照群と比較し生存率が低下した．免疫マウスに対する攻撃前の CGN 処置は生存率の低下を招き，この時の Toxo-GIF 活性はその発現が遅延した．

以上から，Tp 感染に対する防御免疫として感染時の血清抗体あるいは活性化マクロファージの防御に果す役割は低く，リンホカイン等を含む細胞性免疫系がすみやかに応答することが防御に重要であることが示唆された．