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Studies Related to Immunosuppression in Mice with Chronic Toxoplamosis

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ABSTRACT

Mice chronically infected with *Toxoplasma gondii* were treated with cyclophosphamide, obiopeptide-1 (Obi-1) and/or anti-CD4 monoclonal antibody to determine the effect of these immunosuppressive agents on the cysts in the brain. In the brain of non-treated, and infected cyclophosphamide-Obi-1 treated mice, with hematoxylin-eosin, and anti-*Toxoplasma* avidin-biotin-conjugate labelling techniques, large typically rounded tissue cysts were mostly detected, and sometimes with dividing microcysts. In contrast, brain tissue from cyclophosphamide only or anti-CD4 treated infected mice had multiple degenerate cysts of varied size in some brain regions, as well as clusters of microcysts, however, such change was more striking in the anti-CD4 treated group. Infected mice treated with a combination of cyclophosphamide and Obi-1 showed a significantly higher survival of 80% compared to 20% survival in mice treated with cyclophosphamide only. Percent neutrophilic leucocytes, monocytes and lymphocytes in mice treated with a combination of Obi-1 and anti-CD4, or Obi-1 and cyclophosphamide were higher compared to those groups treated with anti-CD4 antibody, or cyclophosphamide only. The increase in neutrophilic leucocyte and lymphocyte counts after a combined cyclophosphamide and Obi-1 treatment may, likewise, contribute to the induction of resistance in mice against *T. gondii*. Furthermore, these results seem to suggest that the reactivation or rupture of tissue cysts in mice chronically infected with *T. gondii* is not principally correlated with the death of cyclophosphamide treated mice.

INTRODUCTION

Within three wk post-infection *Toxoplasma* tachyzoites invade other organs like the brain and muscles of animals forming cysts, with the infection course exhibiting no apparent clinical manifestations. Generally, hosts that are less resistant as a result of acquired immunodeficiency syndrome (AIDS) in humans, distemper in dogs, leukaemia virus infection or administration of immunosuppressive drugs in cancer patients and others, may activate *T. gondii* proliferation within cysts showing clinical signs which can kill the hosts. As to what mechanism(s) activates *T. gondii* to multiply rapidly and form cystozoites, is still not well elucidated. Vollmer et al. (1987) had reported the appearance of *T. gondii* cysts of varied size in the mouse brain, and tachyzoite migration following experimental administration of anti-CD4 antibodies (Ab's). The importance of both CD4 and CD8 positive cells in chronic toxoplasmosis has been likewise, reported by Ricardo et al. (1991), Araujo (1991), and Suzuki et al. (1988).

The present study sought to carry out two objectives. First, was to determine the effect of cyclophosphamide, a widely used immunosuppressant (Makim et al. 1991; Stockman et al. 1973), a drug for cancer treatment with OK-432 (Ogawara et al. 1992), and a drug for the treatment of opportunistic infections in mouse model (Fujii et al. 1992b) on cyst movement in the brain when administered together with anti-CD4 antibody. Second, was to determine the life prolongation effect of synthetic obiopeptide-1 (Obi-1), as an immunoregulator on immunosuppressed mice with chronic toxoplasmosis.

MATERIALS AND METHODS

Experimental host and parasite: Five wk old male BALB/c mice and cysts of *T. gondii* (Beverley strain) maintained at the Department of Veterinary Physiology were used.

Preparation of T. gondii chronic carriers: An emulsion of infected mouse brain was inoculated into the mouse peritoneal cavity. Mice were intramuscularly injected 1 mg/ml/day of prednisolone for 5 days. Seven days later, the peritoneal cavity was washed with 0.9% physiological saline solution (PSS) to obtain tachyzoites. Mice were intraperitoneally (IP) inoculated 1×10^2 tachyzoites, and on the 5th, 7th and 9th day post-inoculation (PI), they were orally administered 0.5 mg/ml of Daimeton (sulfamonomethoxine). Mice that survived the initial infection 28 days PI were challenged with 1×10^4 tachyzoites. Those that survived for 56 days were used as *T. gondii* chronic carriers. Tachyzoites that were used for the challenge dose were obtained from subcultures in ICR-JCL mice which were treated with prednisolone.

Administration of cyclophosphamide and anti-CD4 antibodies: Cyclophosphamide

(Janssen Chimica, Beerse, Belgium) dissolved in 0.9% PSS was IP- inoculated to chronic carrier mice (250 mg/kg) five times at 7 days interval or four times at 10 days interval. In both experiments, the control groups consisted of non-infected mice but treated similarly as the experimental animals. Experimental and control mice were administered 300 μg/head of anti-CD4 monoclonal AB's (mAb's) four times at 10 days interval. Anti-CD4 mAb's were obtained by inoculating GKL.5 cell hybridoma to nude mice, and Ab's were purified from ascitic fluid using ammonium sulfate precipitation.

Administration of Obi-1: Synthetic Obi-1 (Suzuki et al. 1990) was dissolved in 100 mM $NaHCO_3$ (10 mg/ml) pH 6.4, diluted in PSS (1 mg/ml) and sterilized using a micropore filter. Each mouse was injected 100 μg (0.1 ml) of Obi-1 into the femoral muscle on the 3rd and 7th day post-administration of cyclosphosphamide and anti-CD4 Ab's.

Preparation of mouse spleen cells: Spleen cells from infected and non-infected mice were prepared following the method of Igarashi et al. (1990). Mice were bled to death and immediately the spleen were excised, finely cut and suspended in Hanks balanced salt solution (HBSS) containing 5 units/ml heparin, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate, and filtered using a steel mesh. To the sediment obtained by centrifugation at 800xg for 7 min at 4℃, warmed (37℃) 0.83% ammonium chloride solution was added to lyse the erythrocytes. The cells were washed twice with HBSS and once with RPMI-1640 (containing 5 units/ml heparin, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, 12 mM Hepes, 0.1 μM 2-mercaptoethanol) by centrifugation at 800xg for 7 min at 4 ℃.

Calculation of spleen cell subpopulations: Twenty mice were IP-inoculated about 100 tachyzoites/head. At 14, 28, and 162 days PI, five mice per group were used as *T. gondii* - infected group. Unexposed normal mice comprised the control group. Cell density ratio (I/N) was obtained by dividing the cell number in infected by the cell number in the non-infected mice. To the spleen cell suspension Ab's diluted in RPMI 1640 containing 0.3% bovine serum albumin (BSA) was added to approximate 1×10^6 cells/ml, then allowed to stand for 60 min at 4℃ with stirring at 15 min interval. Monoclonal Ab's used were anti-Thy-1,2 (x500), anti-CD8 (x60), and anti-CD4 (x50) (Cedarlen Laboratories Limited, Ontario, Canada) , and Asialo GM1 (x60) (Wako Pure Chemical Industries, Tokyo). The cell suspension was centrifuged and washed once, and rabbit complement (x15 diluted in RPMI 1640 & 0.3% BSA) was added with stirring to approximate a cell density of 1×10^6 cells/ml, then allowed to stand for 60 min at 37 ℃ with stirring at 15 min interval. For the control 1×10^6 cells/ml were used. To the cells, trypan blue was added to approximate a 10% concentration, then placed on ice for 2-3 min, and the ratio of positive cells was obtained following the formula: % dead cells upon addition of Ab's - % dead cells in control divided by 100% - % dead cells in the control.

Calculation of leucocyte count: Heparinized blood obtained from the mouse caudal vein was diluted in Türk's solution using a melangeur. Leucocyte count per mm^3 was noted using a hemocytometer. With Giemsa-stained blood smear, the ratio of neutrophils, eosinophils, basophils, monocytes and lymphocytes to the total leucocyte count was obtained.

Calculation of cyst density in the brain: Using a sharp knife, the intact mouse brain was divided into the right lobe for histological examination, and the left lobe for the calculation of cyst density. To the left lobe, PSS was added and then homogenized using glass slides. Cyst density was calculated by placing 1/20 of the brain tissue emulsion on a plankton counter. A mean value was obtained from three counts and multiplied by 40 to get the cyst number per mouse. The right lobe was fixed in 15% formalin solution, processed and embedded in paraffin. Tissue sections were stained with hematoxylin & eosin (H&E). Parasites in tissues were, likewise, pathohistologically examined with light microscopy. Tissue sections were stained with avidin-biotin-peroxidase, and thereafter, were soaked in 0.3% methanol hydrogen peroxide for 30 min, washed with buffer solution and reacted with goat normal serum for 30 min. Sections were reacted with *T. gondii* - immunized rabbit serum for 30 min, washed and reacted with diluted biotinylated second antibody for 30 min, and with avidin-biotin conjugate (ABC) for another 30 min (Funakoshi Co., Tokyo). After another wash, the sections were reacted with peroxidase substrate solution (0.01% H_2O_2 and 0.05% DAB) (Wako Pure Chemical Industries, Tokyo) and then counterstained with H&E.

RESULTS

Table 1 shows the cell phenotype population ratios (I/N) in both experimental and control (pre-inoculation time) groups. Fourteen days PI, the I/N ratio of $\geqq 2.0$ was noted in total monocytes, Lyt-1,2, Lyt-2,2 and aGM1-positive cells in the spleen. A similar ratio was also recorded with Lyt-1,2, Lyt-2,2 and aGM1-positive cells in the peripheral blood, and in aGM1-positive cells in the liver. At 28 days PI, a ratio of 2.2 for Lyt-2,2 in the spleen and > 2.0 for aGM1-positive cells in the spleen and liver were, likewise, noted. At 162 days PI, the number of Lyt-1,2 and Lyt-2, 2, and aGM1-positive cells in the liver more than doubled compared to the non-infected group.

The effect of Obi-1 in prolonging the survival of chronically-infected mice after the administration of cyclophosphamide is shown in Figure 1. In experiment I (Fig. 1A), all mice in the non-infected control, non-infected-cyclophosphamide treated, and infected control groups survived until the last day of experimentation. With the infected cyclophosphamide-Obi-1 treated mice, one out of 5 died on the 28th day post-treatment (PT), while four out of five died between the 27th and 32nd day PT among the infected cyclophosphamide treated mice. In experiment 2 (Fig. 1B) two

Table 1. Cell phenotype population ratios (I/N) in *T. gondii* (Beverley strain) inoculated mice

	pre-inoculation cell count	Days post inoculation 14 I/N*	28 I/N	152 I/N
Total cells (×10⁶)				
Spleen	13.9	2.1	1.2	1.7
Liver	12.0	1.5	1.2	1.7
Blood	2.5	1.8	1.2	1.1
Thymus	27.3	0.3	0.3	0.7
Thy-1.2(+)cells (×10⁶)				
Spleen	3.9	2.2	1.3	1.9
Liver	5.0	1.5	1.3	1.7
Blood	1.3	1.9	1.2	1.1
Thymus	24.2	0.3	0.3	0.7
sIg(+) cells (×10⁶)				
Spleen	4.0	1.8	1.0	1.9
Liver	0.6	1.6	1.3	1.7
Blood	0.5	1.8	1.0	1.0
Lyt-1.2(+) cells (×10⁶)				
Spleen	2.6	2.5	1.5	1.8
Liver	5.0	1.6	1.5	2.0
Blood	0.2	2.0	1.3	1.0
Lyt-2.2(+) cells (×10⁶)				
Spleen	1.4	2.0	2.2	1.9
Liver	1.8	1.9	1.2	2.6
Blood	0.1	2.0	1.0	1.0
αGM1(+) cells (×10⁶)				
Spleen	1.0	3.0	2.3	1.7
Liver	0.4	2.6	2.2	2.0
Blood	0.1	2.0	1.0	1.0

* Number of cells in infected mice / Number of cells in non-infected mice

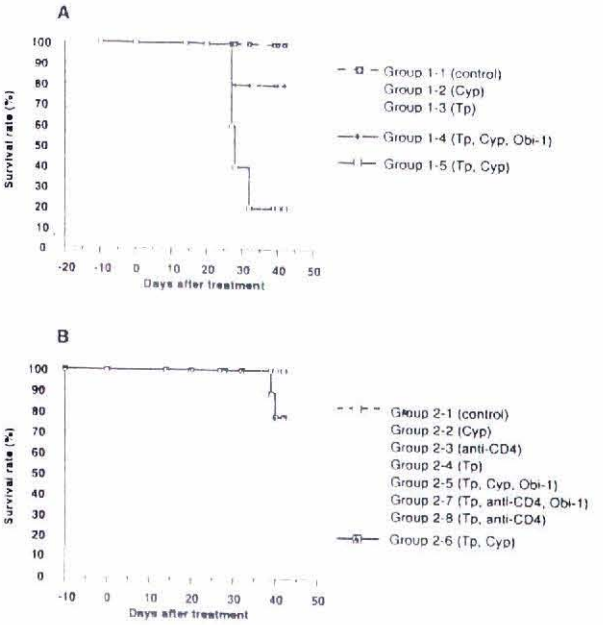

Figure 1. Survival of treated *T. gondii*-chronically infected mice. Mice exposed to 1×10^3 parasites/head and at 28 days post-exposure were challenged with a similar dose. Cyclophosphamide (Cyp) treated at 7 days (Fig.1A) and at 10 days (Fig. 1B) interval. Obi-1 treatment (100ug/head) every 3, 7 days post-Cyp treatment.

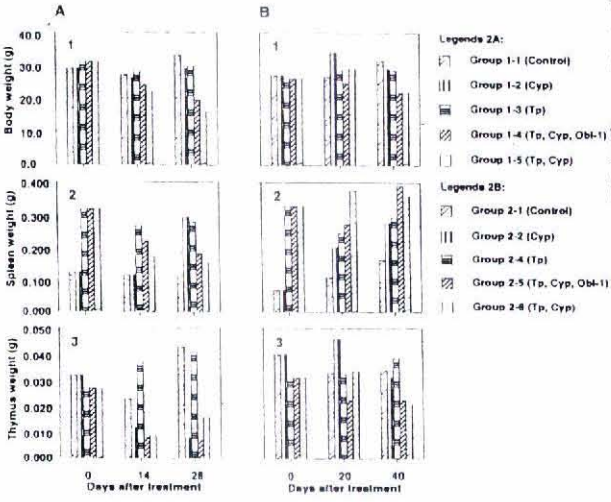

Figure 2. Changes in body (1), spleen (2) and thymus (3) weight of mice chronically infected with *T. gondii* and treated with cyclophosphamide (Cyp), or with Cyp and Obi-1. Treated with Cyp (250mg/kg) at 7 days interval, or with Obi-1 (100ug/head) every 3, 7 days after treatment with Cyp.

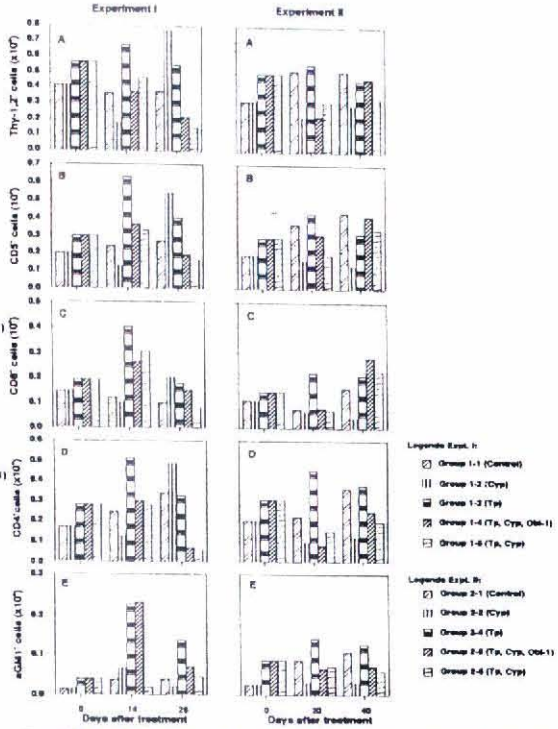

Figure 3. Number of spleen cells in *T. gondii* chronically infected mice treated with cyclophosphamide(Cyp), or with Cyp and Obi-1. Treated with Cyp (250mg/kg) at 10 days interval and with Obi-1 (100ug) every 3, 7 days after treatment with Cyp.

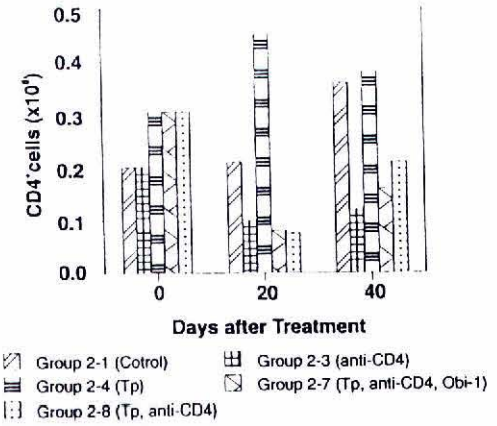

Figure 4. Number of CD4⁺cells in the spleen of mice chronically infected with *T. gondii* and treated with anti-CD4, or with anti-CD4 and Obi-1:
Treated with anti-CD4 (300ug/head) at 10 days interval and with Obi-1 (100ug/head) every 3, 7 days after treatment with anti-CD4.

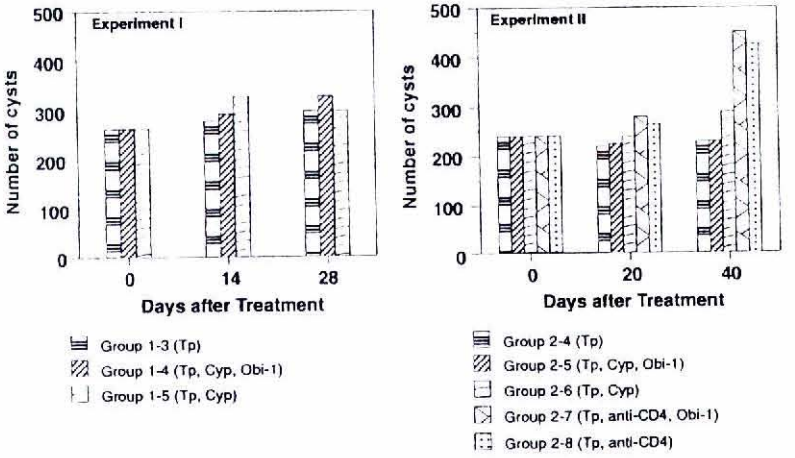

Figure 5. Number of cysts in mouse brain chronically infected with *T. gondii* and treated with cyclophosphamide(Cyp), with Cyp and Obi-1, with anti-CD4 or with anti-CD4 and Obi-1.
Experiment 1: Treated with Cyp(250mg/kg) at 7 days interval, and with Obi-1(100ug/head) every 3, 7 days after treatment with Cyp.
Experiment 2: Treated with anti-CD4(300ug/head), or Cyp(250mg/kg) at 10 days interval; Treated with Obi-1(100ug/head) every 3, 7 days after treatment with anti-CD4 or Cyp.

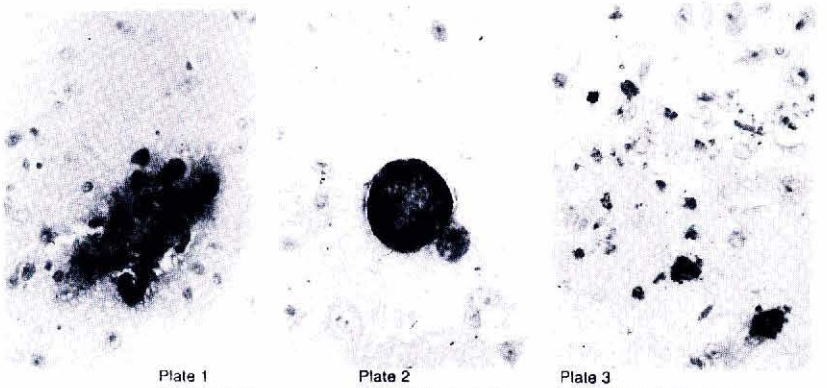

Plate 1 (Cyp treated) | Plate 2 (Cyp+Obi-1 treated) | Plate 3 (anti-CD4 Ab's treated)

Parasites in brain tissues of mice stained with avidin-biotin-peroxidase.

out of five infected mice treated with cyclophosphamide only, succumbed to death on the 39th and 40th day PT ; while Groups 2-1, 2-5, 2-7 and 2-8 had a 100% survival.

Changes in the weight of body and immune organs of mice with chronic toxoplasmosis administered treatments are summarized in Figures 2A and 2B. Infected cyclophosphamide treated, and infected cyclophosphamide-Obi-1 treated mice exhibited weight loss (Figs. A1 & B1). Spleen weight of non-infected cyclophosphamide treated mice increased significantly on the 28th PT; while those of infected cyclophosphamide treated, and infected cyclophosphamide and Obi-1 treated exhibited spleen weight reduction (Fig. A2). In another experiment, however, we noted an increase in spleen weight in Groups 1-4 and 1-5 at 40 days PT. Mice that received cyclophosphamide treatment exhibited reduction in thymus weight compared to the non-treated groups (Fig. A3). A similar pattern is evident in Figure B3.

Figure 3 shows the changes in lymphocyte subpopulation counts in the spleen of *T. gondii*-chronically infected mice given cyclophosphamide and Obi-1 treatment. Results of experiment 1 (Fig. 3) gave consistently higher counts of Thy-1,2, CD5, CD8 and CD4- positive cells in Group 1-3, compared to all other groups at 14 days PT (Figs. A-D). At 28 days PT, Groups 1-4 and 1-5 showed a reduction in Thy-1,2, CD5, CD4, CD8 and aGM1-positive cells, demonstrating a state of immunosuppression. A similar pattern of cell subpopulation reduction was noted in experiment 2, at 20 days PT . At 40 days PT, however, cell counts increased in all groups except in Group 1-2.

The number of CD4- positive cells in mice administered anti-CD4 Ab's is shown in Figure 4. At 20 days PT, infected non-CD4 Ab treated mice showed a significant increase in CD4- positive cells; while those of the non-infected, and infected and Ab treatment exhibited a decrease in CD4- positive cells. A similar pattern was noted at 40 days PT.

Intracerebral cyst density is shown in Figure 5. In experiment 1 at 14 days PT, infected mice treated with cyclophosphamide manifested a marked increase in cysts count compared with the infected (control), and infected cyclophosphamide and Obi-1 treated mice. At 28 days PT, however, infected mice treated with cyclophosphamide and Obi-1 showed an increase in cyst density. In experiment 2, while no significant difference in cyst number was noted between groups at 20 days PT, a significant change in cyts density was observed 20 and 40 days among infected mice administered anti-CD4 Ab's, and among infected mice treated with anti-CD4 Ab's and Obi-1. In cyclophosphamide treated mouse, clusters of small cysts were observed in some brain regions and tachyzoites could hardly be found (Plate 1). However, in cyclophosphamide and Obi-1 treated mice, pathohistological detection of cysts revealed them to mainly possess clear cysts walls, and sometimes with dividing microcysts (Plate 2). Mice administered anti-CD4 Ab's were morphologically similar to those observed in mice treated with cyclophosphamide only. However, some

microcyst exhibited faint cyst wall with H&E. Degenerate cysts of varied size and small cysts in colonies , and some tachyzoite-like organisms were, likewise, observed with ABC staining (Plate 3).

Total leucocyte counts and the ratio of neutrophils demonstrate no marked difference between groups at 20 days PT. At 40 days PT, the non-infected cyclophosphamide treated mice had the lowest white blood cell (WBC) count and percent neutrophil, while those of infected mice treated with cyclophosphamide only , or cyclophosphamide and Obi-1, exhibited higher WBC count, and percent neutrophil, lymphocyte and monocyte ratios.

DISCUSSION

The suppression of all immunologically competent cells including T and B lymphocytes at the stage of DNA synthesis has been reported (Igarashi et al. 1990; Miyauchi et al. 1990; Schwartz et al. 1978; Turk et al. 1972). Ogawara et al. (1992) contend that the administration of cyclophosphamide at a dose of 250 mg/kg can cause rapid reduction of granulocytic and splenic cells and their disappearance in 3-5 days, returning to the normal level within 7 to 14 days, therafter. In the present study, the administration of cyclophosphamide to mice with chronic toxoplasmosis effected a state of depression, loss of body weight and reduction in Thy-1,2, CD5, CD8, CD4 and aGM1 positive cells, as well as atrophy of the thymus and spleen as a result of immunosuppression. In contrast, cyclophosphamide treated non-infected mice exhibited splenoma and a marked increase in Thy-1,2, CD5, CD4 positive cells and intrasplenic nucleated cells.

Intracerebral cysts from non-infected untreated, and non-infected treated groups exhibited numerous intracerebral cysts of similar large size, and with H&E and ABC staining, the cysts showed clear cyst walls. In the treated group, at 28 days PT, cyst density declined. Vollmer et al. (1987) and Mark et al. (1991) had documented the appearance of cysts of varied sizes and sometimes dividing cysts mainly in the brain, as well as the migration of tachyzoites in chronic mice treated with anti-CD4 Ab's. In our study, microscopic examination of brain tissue sections of chronic mice treated with anti-CD4 Ab's revealed faint cyst walls containing degenerate irregular cysts undergoing fission and clusters of small cysts. In infected cyclophosphamide treated mouse brain most of the cysts were of large size, nonetheless, in some regions there were degenerate irregular small cysts in clusters. However, tachyzoite migration was hardly observed. Furthermore, the non-infected control, and infected cyclophosphamide and Obi-1 treated groups showed typical intracerebral cysts with clear or sharp cyst walls, and sometimes mutiple dividing cysts of various sizes.

Monitoring the movement of intrasplenic T- lymphocyte subpopulations with the

use of their specific Ab's revealed a reduction in Thy-1,2, CD5, CD8, CD4 and aGM1-positive cells in non-infected mice as immunosuppression progressed with cyclophosphamide administration. In contrast, among chronically treated infected mice, there was no marked change in the values of lymphocyte subpopulations, however, the number of CD4 cells was significantly low, about half the density noted among clyclophosphamide untreated mice. Mice with chronic toxoplasmosis and administered cyclophosphamide exhibited loss of weight and depression. The authors tend to suppose that the rupture of intracerebral cysts in likely caused by a reduction in CD4 positive cells more than the effect of a reduction of all immunocompetent cells, including T and B- lymphocytes.

Obi-1 has been shown to inhibit T. *gondii* growth in cultured cells of bovine peripheral blood monocytes, mouse intraperitoneal macrophages and renal cells, human myocardial and cerebral cells, and exhibits a non-specific immunoregulating effect on tumours (Sakurai et al. 1982; Suzuki et al. 1990). In the present study, we noted a marked difference in survival rate between cyclophosphamide and Obi-1 treated, and Obi-1 untreated mice. The regular administration of cyclophosphamide to mice with chronic toxoplasmosis may not strongly activate the cysts to proliferate, and percent neutrophilic leucocytes, monocytes and lymphocytes in mice treated with Obi-1 were of higher values compared to those obtained from mice treated with cyclophosphamide only. These observations demonstrate the possible role of Obi-1 in the presence of cyclophosphamide, in prolonging the lifespan of chronically infected mice. From these results, we tend to think that the difference in host resistance and/or a difference in host life threshold limit against the fatal effect of acute toxoplasmosis could have been influenced by a leucocyte count below the normal lower threshold value, which is otherwise needed for survival. Fujii et al. (1992b) reported enhanced susceptibility of mice administered cyclophosphamide to bacteria and such susceptibility correlated clearly with a reduction in leucocytes. In the present study, the ratio of neutrophils and monocytes to total WBC count was highest among mice treated with cyclophosphamide and Obi-1. Fujii et al. (1992b) and Kono et al. (1992) have shown that the addition of Obi-1 does not only reverse the effect of leucocyte reduction due to cyclophosphamide, but Obi-1, also, accelerates phagocytic bactericidal activity by functioning as a hematopoeitic cell stimulating-like factor in a host under a state of immunosuppression. It can be speculated, moreover, that there could be a factor influencing the difference in effect between cyclophosphamide and Obi-1, thus influencing the increase in number and activation of neutrophils, lymphocytes and monocytes.

The administration of anti-CD4 Ab's to mice with chronic toxoplasmosis resulted to a reduction in CD4 positive cells in the spleen, irregular sized cysts undergoing fission, and small cysts in colonies scattered in the brain. Furthermore, the administration of

cyclophosphamide alone to chronic mice also caused a decrease in CD4- positive cells, however, immediately after fission, irregular sized cysts showed less obvious changes compared with those noted among anti-CD4 treated mice. As to how anti-CD4 Ab's cause the disruption and multiplication of intracerebral cysts, and as to what mechanism(s) is involved in reducing CD4 positive cells in the presence of cyclophosphamide or of any other immunosuppressive agents are questions that warrant further investigations.

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