Coccidia are a group of protozoan parasites which are very common and infect dogs, cats, rabbits, rats, mice, horses, cattle, goats, sheep, chickens and many other species of animals, as well. The coccidians that infect pets include *Eimeria*, *Isospora*, *Hammondia*, *Toxoplasma* and *Neospora* species. Of the infections caused by these species, two that are usually referred to as “coccidiosis” are *Eimeria* and *Isospora* infections. The clinical signs of poor weight gain, diarrhea ranging from mucoid, watery to hemorrhagic, polydipsia and sometimes acute death are seen in young animals including puppies, kittens and rabbits as well as in poultry by *Eimeria* infection.

Many kinds of chemotherapy are extensively used to control coccidiosis, but against a part of the currently available chemical agents the resistance by pathogens is prevalent [2, 3, 19], and the establishment of alternative chemotherapeutic agents effective against coccidiosis is needed. On the other hand, *Eimeria*-mouse model has been used to investigate the immunological problem in mammalian coccidiosis. Chemical inhibition of the proliferation of parasites is a useful method to see the specific immune responses to the different developmental stages of parasite, however, it is difficult to induce the protective immunity in the *Eimeria*-mouse model because of a lack of fully effective chemicals against the infection in mice. The lincosamide antibiotic, clindamycin, is active against apicomplexan parasites such as *Plasmodium*, *Toxoplasma*, *Babesia*, and the fungal pathogen *Pneumocystis* spp. [14]. Clindamycin is the drug of choice for prophylaxis of *Toxoplasma* chorioretinitis in newborn infants [20] who exhibit toxicity to standard treatment with pyrimethamine/sulphadiazine [12] and is part of the regimens recommended against both *Babesia microti* and *B. divergens* [6]. To date, the anti-eimerian effects of clindamycin have not been reported. In the present study, we determined the therapeutic effect of clindamycin to *E. pragensis* infection in a mouse model and assessed the interaction between treatment schedule and developing protective immunity.

**MATERIALS AND METHODS**

**Animals:** Male C57BL/6 mice, 8–9 weeks old with around 25 g body weight (BW) were purchased from Clea & Shizuoka Experimental Animal Supplier Co., Japan, housed in clean metal cages and fed with a standard diet and tap water *ad libitum* in an air-conditioned room (23 ± 1°C), under conventional conditions with a 12:12 hr, light: dark cycle. They were kept as outlined in the “Guide for the Care and Use of Laboratory Animals” by The Faculty of Agriculture, Miyazaki University.

**Parasites:** The pathogenic agent used in this study was *E. pragensis* [1] obtained from the Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire RG20 7NN, U.K., and routinely maintained in our laboratory by oral passage through C57BL/6 mice [17].

**Drug:** The drug used was clindamycin phosphate “Dalcin S” (Pharmacia Co., Tokyo). Clindamycin was injected subcutaneously at doses calculated per kg BW on the basis of human therapeutic dosages.

**Experimental procedures:** All infective doses of *E.
**RESULTS**

**Dose-dependent efficacy of clindamycin:** Statistically significant reductions in oocyst output were obtained by the treatment at doses of 400 mg/kg/day and 800 mg/kg/day compared with those in unmedicated mice (Fig. 1). Treatment at dose of 800 mg/kg/day completely inhibited oocyst output, whereas that at a dose of 400 mg/kg/day reduced output by approximately 75%. Mice treated either at 50 or 100 mg/kg/day produced the similar numbers of oocysts to those of unmedicated mice (Fig. 1).

**Effect of treatment schedule on oocyst output:** The data obtained in Exp. 2 confirmed the almost complete and incomplete effectiveness of the treatment at a dose of 800 mg/kg/day for 12 days and from day 1 to day 4 or day 4 to day 8, respectively (Fig. 2), that is, untreated mice produced 2.57 ± 5.12 × 10⁶ oocysts, whereas those treated for 12 days [0.09 ± 0.02] × 10⁶ oocysts (Group 1, p<0.001), those treated from day 1 to day 4 [5.62 ± 1.27] × 10⁶ oocysts (Group 2, p<0.01) and those treated from day 4 to day 8 [1.38 ± 0.64] × 10⁶ oocysts (Group 3, p<0.01). The total oocyst output obtained from the mice treated by a day 8 to 12 treatment schedule (Group 4, [13.61 ± 4.29] × 10⁶) was not significantly different from that obtained from the unmedicated mice (Group 5).

**Protection against challenge infection of mice treated with different treatment schedules in the primary infection:**
The primary infection of the treated or untreated mice induced protective immunity against challenge infection, so that the total oocyst outputs during challenge infection were quite lower than those during the primary infection (Fig. 3). Interestingly, the different schedules of treatment with clindamycin in the primary infection produced different degrees of protective immunity against the challenge infection. Specifically, the mice that treated for 12 days produced more oocysts than those in the other treated group and those in the unmedicated (DW 1–12, Group 5) during the challenge infection, although the oocyst production by this group was quite lower than that untreated in the primary infection. Mice that treated from day 1 to 4 (Group 2), day 4 to 8 (Group 3) or day 8 to 12 pi (Group 4) produced similar numbers of oocysts to those of the mice that were unmedicated during the challenge infection, that is, [2.03 ± 1.75] × 10⁶, [3.54 ± 2.59] × 10⁶ and [0.08 ± 1.36] × 10⁶ compared with [1.54 ± 2.67] × 10⁶. Although the group treated on day 8–12 pi showed the lower mean level of oocyst output than those treated from the day 1 to 4, day 4 to 8 pi or the untreated group, this difference in oocyst output of these latter groups was not statistically significant.

Effect of the treatment on the clinical symptoms during the primary and challenge infection of treated mice: Mice that received the 12 days treatment (Group 1) showed no clinical symptoms in such as fecal consistency, appetite and activities. Then, mice treated from days 1 to 4 (Group 2) and/or days 4 to 8 pi (Group 3) appeared healthy and active, soft feces were seen at days 8 and 9 pi. Mice treated from 8 to 12 days pi (Group 4) began to have diarrhea on day 7 pi, which progressively contained blood on days 8 and 9. These conditions appeared with depression, anorexia and dullness. Clinical conditions of the unmedicated mice (Group 5) were not significantly different from those of Group 4. When the mice treated for 12 days (Group 1) were challenged, diarrhea, anorexia and moderate weakness occurred at days 7 to 9. In contrast, after challenge infection, the mice treated for days 8 to 12 (Group 4) showed no clinical symptoms as well as the unmedicated group of mice (Group 5). Specifically, the mice that received the treatment days 1 to 4 (Group 2) or days 4 to 8 pi (Group 3) showed clear reduction in clinical symptoms as well as protection to challenge infection. Moreover, no adverse side effects were demonstrated by the treatment with clindamycin in E. praegensis infected mice.
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Morphological comparison of the endogenous stages of *E. pragensis* between treated and untreated infected mice:

In order to determine the effects of clindamycin treatment on the endogenous development, samples of tissues were taken from treated and untreated mice 3 days pi and at 6 days pi. The period of three days pi is the time when the second generation schizogony is in progress and most of the second trophozoites and immature schizonts were morphologically degenerated in the mice treated 1–4 days pi, whereas in the untreated group, the stages of parasite were histologically normal as well as the multinucleated immature schizonts (Figs. 4–A and B). In the 1 to 4 day-treated mice, a few 1st-generation schizonts remained but morphologically degenerate. In contrast, numerous mature 1st-generation schizonts were observed within the epithelial cells of untreated mice (Figs. 4–C and D). In the treated (Clm 1–4) mice, most of the degenerate immature 2nd-generation schizonts had coarse granular cytoplasm and showed poor

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**Fig. 4.** Morphological comparison of endogenous stages of *E. pragensis* between Clm treated (A, C, E, G and I) and untreated (B, D, F, H and J) mice at doses of 800 mg/kg/day. Fixed in 10% Neutral Buffered Formalin and stained with H & E. Magnification × 1,000. Abbreviations: tr, trophozoite; rb, residual body; mr, merozoite; sch. 1., 1st-generation schizont. A and B: The tr (arrows) of 2nd-generation schizont in the crypt epithelial cells of treated and untreated mice 3 days pi; tr in treated mice appeared degenerated (Clm 1–4)(A), while tr in untreated mice developed to be multinucleated (B). C and D: Mature sch. 1 (arrows) in the epithelial cells of mice 3 days pi; The sch. 1 with pycnotic mr were seen in treated (Clm 1–4) mice (C), while sch. 1 in untreated mice had numerous mr (D). E and F: Immature sch.2 (arrows) 3 days pi; The sch.2 in treated (Clm 1–4) mice appeared shrunken and lacks nuclear differentiation (E), whereas sch.2 of untreated mice were well differentiated and multinucleated (F). G and H: Mature 3rd-generation schizont 6 days pi had mr and a residual body lying free within the parasitophorous vacuole (arrow); The mr of treated (Clm 4–8) mice was pycnotically degenerated (G), while mr in untreated mice was well developed (H). I and J: At 6 days pi, sch. 4 of treated (Clm 4–8) mice had a few marginal nuclear remnants (I), whereas mature sch. 4 with a centrally located rb surrounded by numerous small mr were encountered in untreated mice (J).
nuclear differentiation, which made their appearance distinctly different from the normal immature schizonts seen in the untreated mice at 3 days pi (Figs. 4- E and F). Occasionally, at 6 days pi, mature 3rd -generation schizonts had merozoites with a residual body lying free within the parasitophorous vacuole. The mature schizonts in the treated (Clm 4–8) mice exhibited pycnotic degeneration, while the merozoites in a mature schizont were well developed in the untreated mice (Fig.4- G and H). Moreover, in the clindamycin-treated mice, the 4th-generation schizonts had a few nuclear remnants at the margin, whereas the mature 4th-generation schizonts with a centrally located residual body surrounded by numerous small merozoites were observed in the untreated mice (Figs. 4- I and J) [16, 17].

DISCUSSION

In the present study, clindamycin treatment was very effective at doses of 800 mg/kg/day for 1 to 12 days pi for reducing the total oocyst production and clinical symptoms in murine coccidiosis, but this treatment schedule did not induce a high degree of protective immunity in the treated mice. In contrast, clindamycin had a moderate anti-coccidial effect, when administered at a doses of 800 mg/kg/day for the shorter periods from 1 to 4 or 4 to 8 days pi of schizogony, but some parasites completed the life cycle and the level of immunity equivalent to that obtained by the late treatment schedule from day 8 to 12 and/or in the mice unmedicated in the primary infection. Therefore, the shorter period of treatment from 1 to 4 days or 4 to 8 days pi resulted in a significant reduction in the total oocyst production and an improvement in the clinical conditions.

The short-period treatment made in the early course of infection (day 1 to 4) was targeted to the early developmental stages of parasite (the 1st and 2nd - generation schizogony), whereas the intermediate (day 4 to 8) period of treatment would affect the later developmental stages of endogenous parasite. However, the late schedule of treatment (day 8 to 12) would only affect the very late stages of endogenous parasite since they would have completed the life cycle and were released into the lumen and passed out of hosts with the feces. The endogenous phase of eimerian life cycle starts when sporozoites penetrate the enterocytes and the infection can be diagnosed by the appearance and location of the developing parasites of the Eimeria spp. With E. pragensis, the 1st-generation trophozoites and immature schizonts are principally located in the cecum and colon approximately 18 hr pi and developed up to the first mature 4th-generation schizonts at 6 days pi [16]. The parasitic stages that we observed in the sections taken at days 3 and 6 pi were identical with those previously described [16]. Histological examination of the gut of infected and infected/treated mice revealed the dramatic changes in the number and morphology of developing parasites. It is worth noting that, in vitro cultivated T. gondii, clindamycin affects the proliferation of intracellular parasites only in the 2nd parasitophorous vacuole, that is, not affecting immediately after application of the drug [5]. Surprisingly, most apicomplexan parasites including Eimeria coccidians possess a non-photosynthetic chloroplast, termed the apicoplast [4], which might serve as the targets for the development of drug against apicomplexa [23].

All the short-term treatment schedules were effective to generate immunity against challenge infection as well as that obtained without treatment, which resulted in the dramatic reduction in oocyst output. These data indicate that antigen load reduced by removing parasite stages in the primary infection had little effect on the degree of immunity generated in the primary infection of mice with E. pragensis. Moreover, the level of immunity generated in the primary infection was similar although the endogenous stages of parasite were reduced in number by treatment with the drug at different stages of the life cycle and this suggests that protective antigens may be encoded in multiple stages of parasite. Early studies made in the differently timed treatment schedules with sulfonamide of E. tenella infection of chickens revealed that development of the 2nd - generation schizont was necessary before protective immunity was developed [7, 13]. Thus, for E. tenella infection, at least the partial development of the 2nd-generation schizont stage, which is the large, deeply seated and pathogenic stage, is necessary to induce the protective immune response. The previous study made by the combination method of drug-arrested development and transfer of infective mucosal preparations concluded that the 2nd-generation schizogenic stages were most important to stimulate protective immunity against E. maxima [18]. In contrast, studies reported that sporozoites could induce substantial protective immune responses although related with some intracellular metabolism [8–10], and gametocyte preparations were also shown to induce immunity to challenge [21, 22]. As described above, the previously published data suggest that various developmental stages of parasite can induce substantial immunity. Interestingly, the previous study indicated that larger doses of irradiated E. maxima were required to induce immunity in chickens and suggested that dose of antigen might be important, where the important protection-inducing antigen would affect in the successive developmental stages [11]. Our data allow to put a similar interpretation on E. pragensis but also suggests that the proportions of different developmental stages of parasite may not dramatically influence the development of immunity.

In summary, we described an in vivo anti-eimerian effect of the antibiotic drug, clindamycin, at an effective dose similar to that recommended for the treatment of toxoplasmosis. The short-term treatment schedules which targeted the early and late endogenous stages of parasite are effective not only to reduce the total oocyst output and to improve the clinical symptoms but also to induce the protective immunity against the challenge infection equivalent to that induced by exposing mice to the full life cycle of parasite, that is, not intervened by drug administration. Clindamycin was thought to be useful in controlling coccidiosis in pet animals, including dogs, cats, rabbits, rats, mice, and birds.
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