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Assessment of the growth inhibitory effect of gellan sulfate in rodent malaria *in vivo*

Frances C. Recuenco[1, 2)], Ryo Takano[1)], Tatsuki Sugi[1, 2)], Hitoshi Takemae[1)], Fumi Murakoshi[1, 2)], Akiko Ishiwa[1, 2)], Atsuko Inomata[2)], Yukiko Enomoto-Rogers[3)], Noreen Grace V. Fundador[3)], Tadahisa Iwata[3)], Taisuke Horimoto[2)], Hiroomi Akashi[2)] and Kentaro Kato[1, 2,*)]

[1)]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080–8555, Hokkaido, Japan

[2)]Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan

[3)]Laboratory of Science of Polymeric Materials, Department of Materials Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan

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Abstract

In our effort to discover novel antimalarials, we previously synthesized gellan sulfate, a novel anticoagulant derivative of gellan gum and showed that it inhibits the growth and invasion of erythrocytes by *Plasmodium falciparum* parasites *in vitro* (Recuenco *et al.* 2014. Sci Rep. 4:4723.). Gellan sulfate was also found to have low cytotoxicity and low anticoagulant activities *in vitro*. Here, we assessed gellan sulfate for its antimalarial effects *in vivo*. Despite its effectiveness *in vitro*, gellan sulfate inhibited the growth of both *P. berghei* ANKA and *P. yoelii* 17XL poorly in C57BL/6 and BALB/c mice, respectively. To our knowledge, this is the first work that uses gellan gum and gellan sulfate for intraperitoneal injection in mice.

Key Words: Gellan sulfate, *in vivo*, rodent malaria

Current antimalarial drugs act on intra-erythrocytic *Plasmodium* parasites[21)]. Quinine and artemisinin, two of the more widely used antimalarials, are both derived from plant sources. Both have synthetic derivatives that make them readily available commercially[6)], and both act on malaria parasites by interfering with the uptake and digestion of hemoglobin[9,18)]. Artemisinins are currently the preferred treatment for severe malaria[9)] and artemisinin-based combination therapies (ACTs) are recommended for uncomplicated malaria[3)]. However, the development of resistance[3)] against antimalarials is hindering efforts to eradicate malaria worldwide. Hence, the continuous effort to develop new vaccines and novel drugs is needed.

Heparin is a highly sulfated polysaccharide that binds to several proteins of malaria

*Corresponding author: Dr. Kentaro Kato, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080–8555, Hokkaido, Japan
Phone: +81–155–49–5645. Fax: +81–155–49–5646. E-mail: kkato@obihiro.ac.jp
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parasites, inhibiting parasite entry into red blood cells *in vitro*[21]. Heparin can also inhibit cytoadherence and sequestration of infected red blood cells to the vascular endothelium[22] and its low molecular weight derivatives have been shown to prevent rosetting *in vitro*[17]. However, heparin is not safe for use in clinical malaria because it can cause intracranial bleeding[20]. Other sulfated polysaccharides, including dextran sulfate[22], carrageenans[1], and fucoidan[4] have been found to inhibit parasite entry into red blood cell *in vitro;* but, their effectiveness *in vivo* is still questionable[2,4,7]. In this case, heparin derivatives may represent a clinically safer alternative to heparin. Depolymerized heparin glycans that lack anticoagulant activity have been shown to release sequestered *P. falciparum*-infected red blood cells in the non-human primate model, *Macaca fascicularis*[19]. For prevention of parasite entry into red blood cells, blood-stage antimalaria vaccines are still currently being designed and developed[11].

We previously showed that gellan sulfate, a derivative of the microbial polysaccharide gellan gum, effectively inhibits malaria parasite entry into red blood cells and growth *in vitro*, is non-cytotoxic to 293T cells, and shows weak anticoagulant activity *in vitro*[14]. Here, we assessed the *in vivo* effect of gellan sulfate using mouse models of severe and cerebral malaria. C57BL/6 mice were infected with *Plasmodium berghei* ANKA while BALB/c mice were infected with *P. yoelii* 17XL. Infected mice were treated intraperitoneally with native gellan gum (GG) or gellan sulfate (SGG) following the four-day suppressive test. Gellan sulfate and native gellan gum showed poor growth inhibition of *P. berghei* ANKA and *P. yoelii* 17XL *in vivo*. Nevertheless, to our knowledge, this is the first work to use gellan gum and gellan sulfate for intraperitoneal (IP) injection in mice. At doses of 20–25 mg/kg, no apparent signs of adverse reactions to IP GG and SGG were observed based on subjective visual assessment. These additional observations still warrant further investigations but may be useful to consider for conducting future studies involving systemic GG and SGG administration.

Frozen stocks of *P. berghei* ANKA and *P. yoelii* 17XL parasites were passaged in C57BL/6 mice and BALB/c mice, respectively. Five-week-old, female C57BL/6 and BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). The animals were maintained in the animal care facility at the National Research Center for Protozoan Diseases under controlled conditions and were given commercial feed and water *ad libitum*. The protocol for the animal experiments was approved by the Committee on Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit No. 25-153). Donor mice were infected intraperitoneally with 1×10^6 parasitized red blood cells from cryopreserved stock. *P. berghei* ANKA-infected blood from the C57BL/6 donor mice were collected at 6% parasitemia before the presumed onset of cerebral malaria. *P. yoelii* 17XL-infected blood from donor BALB/c mice were collected at 15% parasitemia.

Artesunate (SIGMA) was dissolved in 1% sodium bicarbonate in PBS (2 mg/ml) and filter-sterilized by passage through a 0.45-μm filter. Gellan gum (WAKO) and gellan sulfate were both dissolved in PBS (2 mg/ml) and similarly filter-sterilized. Since gellan gum gels in solution, it was first heated to 65°C before filtration. Gellan sulfate was prepared by mixing 1% (w/v) gellan gum with 0.1 M HCl to pH 3.0 and confirming successful sulfation by nuclear magnetic resonance analysis, as described previously[14].

Experimental mice were infected intraperitoneally with 1×10^6 parasitized red blood cells and were divided into four treatment groups: untreated control, artesunate-treated (ART), gellan gum-treated (GG), and gellan sulfate-treated (SGG). Treatment and monitoring were performed by following the four-day suppressive test protocol. GG and SGG were given intraperitoneally at doses of 20 mg/kg for C57BL/6 mice and 25 mg/kg for BALB/c mice. Artesunate was given at 20 mg/kg. Parasitemias

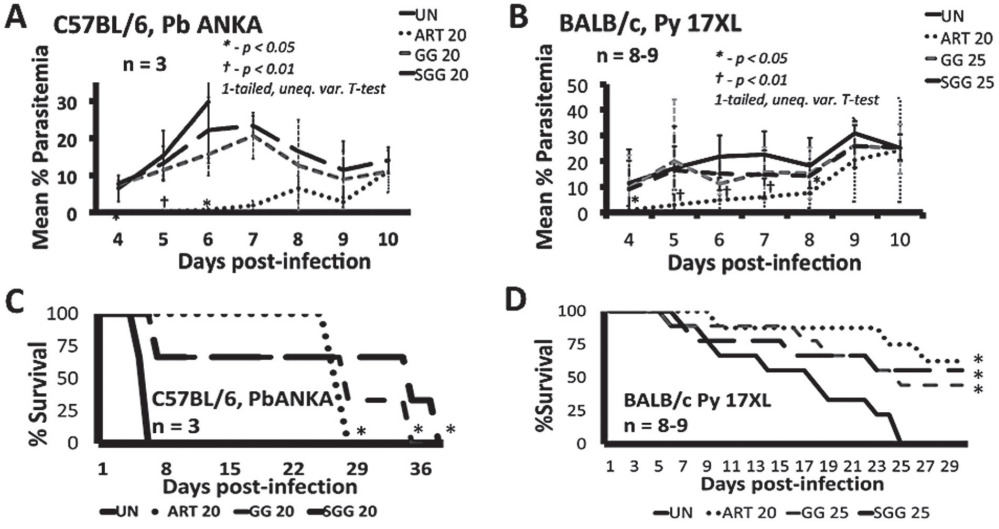

Fig. 1. Parasitemia and survival monitoring of *P. berghei* ANKA-infected C57BL/6 mice and *P. yoelii* 17XL-infected mice treated with 20–25 mg/kg gellan gum or gellan sulfate. C57BL/6 mice (n = 3 per group) and BALB/c mice (n = 8–9 per group) were infected intraperitoneally with 10^6 *P. berghei* ANKA- and *P. yoelii* 17XL-infected red blood cells, respectively. Groups of mice were treated with artesunate (20 mg/kg, ART 20), gellan gum (20 mg/kg, GG 20; 25 mg/kg, GG 25), or gellan sulfate (20 mg/kg, SGG 20; 25 mg/kg SGG 25), intraperitoneally, following the 4-day suppressive test protocol. (**a, c**) Parasitemia was monitored by examining Giemsa-stained thin blood smears from day 4–10 p.i.. One-tailed T-tests were performed to determine significant differences between control and treated groups. (**b, d**) The graphs show the % survivals of the infected mice. Asterisk: P value < 0.05, significant difference compared to UN, untreated group (log-rank test).

were monitored from day 4 until day 10 post-infection (p.i.) by examining Giemsa-stained thin blood smears. Ten oil immersion fields with 200–300 cells were counted, except for parasitemias approaching zero, in which cases, 100 fields were examined. Mice were observed for changes in appearance and behavior for possible signs of adverse reactions to treatment. We performed one-tailed t-tests for samples with unequal variances to compare the parasitemias of the treated groups to the untreated control groups with significance levels, $p < 0.05$ and $p < 0.01$[5,10]. For the BALB/c mice infected with *P. yoelii* 17XL, we pooled data from two independent experiments. Deaths were recorded promptly. Statistically significant differences in mouse survival of ART-, GG-, or SGG-treated groups compared with untreated group were determined by using Log-rank test; values were considered to be significantly different when the P value was less than 0.05.

The parasitemias of the C57BL/6 mice infected with *P. berghei* ANKA and BALB/c mice infected with *P. yoelii* 17XL treated with GG and SGG were not significantly different from those of the untreated control groups (Fig. 1A and B) based on one-tailed t-tests. By contrast, there were significant differences between the parasitemias of the untreated control and the artesunate-treated mice (Fig. 1A and 1B).

All *P. berghei* ANKA infected control mice died by day 6 p.i., compared to just one death in each of the SGG- and GG-treated groups on day 7 p.i. (Fig. 1C). Artesunate-treated mice also succumbed to the infection by day 26–28 p.i. and the last SGG- and GG- treated mice died on days 35 and 38 p.i., respectively (Fig. 1C). There was 50% mortality for the *P. yoelii* 17XL infected control mice by Day 15 p.i (Fig. 1D). At Day 25 p.i., all control mice have died and about 50% of both GG and SGG treated mice (Fig. 1D). Half of the artesunate treated mice died by Day 29 p.i (Fig. 1D). From this point, the surviving mice from the GG, SGG, and ART treatment groups were eventually sacrificed on Day 60. Parasitemias at the time of deaths were not collected and

pathological tests were also not done this time. We statistically analyzed the mouse survival using Kaplan-Meier method followed by Log-Rank test. In both mouse malaria models, we found statistically significant differences ($P < 0.05$) in the survival of mouse treated with ART, GG, or SGG compared with those untreated (UN), indicating that GG and SGG have life-prolonging effects in these murine malaria models.

Several factors can affect the effectiveness of drugs in animals. The animal's immune response to the disease itself is a major determinant of the course of the disease and response to treatment. In addition, the method of administration and the delivery system can affect the absorption and metabolism of drugs. The deaths in the *P. berghei* ANKA- and *P. yoelii* 17XL-infected groups treated with artesunate may have been due to the limited time of treatment. Oral artesunate given at 10 mg/kg/day for 7 days has been shown to prevent parasitemia but with the likelihood of recrudescence of parasitemia and development of cerebral malaria[6]. A 14-day treatment course of oral artesunate at 100 mg/kg/day completely prevented the development of parasitemia and cerebral malaria[6]. In this case, artesunate did indeed effectively inhibit the growth and proliferation of the parasites during the early stages of the infection under drug pressure. However, when treatment ended and the parasites were allowed to proliferate and the disease was allowed to run its course, some artesunate-treated mice died within 30 days of infection.

Gellan gum is currently used as drug delivery system, and is said to be safe when given orally. When mixed in the diet of Swiss Crl mice for 96–98 weeks, gellan gum has no effect on the body weight or food consumption of the animals, and no neoplastic or non-neoplastic changes associated with feeding gellan gum were observed[16]. When implanted in the dorsal region of BALB/c mice for 21 days, 1% gellan gum discs causes no significant lesions[13]. However, the nutritive or therapeutic benefits of gellan gum have not been reported. Gellan sulfate, the sulfated derivative of gellan gum, was originally developed as a novel anticoagulant[12]. To our knowledge, this is the first study where gellan gum and gellan sulfate were administered intraperitoneally in mice. We tried to administer gellan gum and gellan sulfate at 50 mg/kg to C67BL/6 mice but observed signs of probable adverse reactions, such as hunching and huddling after injection. Mice given this dose appeared smaller in size and unkempt based on visual assessment. Although some of these changes may have been due to the ongoing disease, these signs were only observed during the four-day treatment period. In contrast, these signs were not evident in mice given lower doses of SGG and GG, suggesting that the dose of 50 mg/kg for intraperitoneal injection in mice may not be suitable; accordingly, it was discontinued and not used on BALB/c mice.

Despite the effective inhibition of growth and invasion of *P. falciparum* 3D7 and Dd2 *in vitro*, gellan sulfate appears to be less effective in inhibiting the *in vivo* growth of the rodent malaria parasites, *P. berghei* ANKA and *P. yoelii* 17XL, based on the parasitemia levels after treatment. Although statistical analyses indicate life-prolonging effects in both murine malaria models with SGG and GG treatments, the reasons for these results remain unclear. It is important to consider the small sample size used in this study and be cautious with the conclusions. We can hypothesize that SGG or GG may have effects on immunity or on sequestration[19] of *P. berghei* ANKA in the brain microvessels. In order to understand this, however, the mechanisms how gellan sulfate and gellan gum behave in the animal body after systemic administration must be further investigated.

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