Studies on the effects of sulfated polysaccharides on malaria infection

（マラリア感染への硫酸化多糖類の効果に関する研究）

Recuenco, Frances Cagayat

レクエンコフランセスカガヤット

CONTENTS

PREFACE

Aims and scope of the thesis

Malaria is one of the most significant parasitic diseases in the world. Even with increased efforts focused on controlling the protozoan parasites as well as the mosquito vector, malaria still claims 600,000 lives and affects 2 million people globally.

Currently, artemisinins, in combination with other antimalarials, are the treatment of choice for severe malaria. However, emerging resistance to artemisinins must be overcome. Vaccines for prevention of infection would be very promising, but it is still currently under testing.

Novel compounds are continuously being screened as potential antimalarials. High-throughput screening technologies, as well as genomic revolution have been instrumental in accelerating the continuing efforts to search for new antimalarials. Target compounds include naturally-occurring and synthetic compounds, as both quinine, which is considered the oldest antimalarial, and artemisinin, were derived from plant extracts. Extracts from plants in endemic areas are also very promising sources of new compounds.

Extracts from marine sources like seaweeds, algae, and macroalgae are currently gaining popularity for their potential medical applications. Sulfated polysaccharides like carrageenans and fucoidan, as well as, new classes of macrolides like diterpenes and bromophycolides have been shown to have antimalarial activities *in vitro*.

In this study, the author describes the assessment of novel polysaccharides for their antimalarial effects *in vitro* and *in vivo*.

The GENERAL INTRODUCTION provides the background on the etiologic agents of malaria, the *Plasmodium* parasites, their life cycle and the pathology of malaria. It also touches on the approaches to the treatment and control of malaria, the current therapies and the challenges that must be faced. It also provides information on the polysaccharides, namely, κ- and λ-carrageenans, gellan gum and gellan sulfate, that were used in these studies.

In CHAPTER 1, the author describes the synthesis of gellan sulfate, the sulfated derivative of gellan gum, oversulfated κ-carrageenan and hydrolyzed λ-carrageenan, the determination of the levels of sulfation of gellan sulfate and oversulfated κ-carrageenan, and the *in vitro* assessment of their activities against *P. falciparum* and their cytotoxicity to 293T cells. The anticoagulant activity of gellan sulfate, a novel anticoagulant was also assessed using APTT assays *in vitro*.

In CHAPTER 2, the author assessed the *in vivo* effect of gellan sulfate on the growth of *P. yoelii* 17XL and *P. berghei* ANKA in mice following the 4-day suppressive test.

In CHAPTER 3, the author describes the endeavor to induce and confirm the development of experimental cerebral malaria by administration of λ-carrageenan in the BALB/c mouse infected with *P. berghei* ANKA.

The GENERAL CONCLUSION discusses the thesis collectively and provides insights for future studies.

GENERAL INTRODUCTION

The etiological agents of malaria, the *Plasmodium* parasites, are obligate intracellular protozoa of the Apicomplexan family. Members of this family of protozoans have the characteristic apical complex that possesses organelles involved in the invasion of host cells. *Plasmodium* species require mosquito primary hosts for sexual reproduction and vertebrate hosts for asexual reproduction and production of the gametocytes. The female *Anopheles* mosquito also serves as vector that transmits the parasites to humans, non-human primates and rodents, while other species of mosquitoes like *Culex* spp. transmit the parasites to birds and rodents.

The human malaria parasite, *Plasmodium falciparum*, is transmitted during the blood meal of an infected female *Anopheles* mosquito (Fig. 1). *P. falciparum* sporozoites in the saliva of the mosquito are injected into the dermis. Some sporozoites are transported to the liver via blood circulation, some are ingested by macrophages, while others go to the lymphatics, ending up in the spleen where they are presumably cleared. Those that have migrated to the liver invade hepatocytes and produce daughter merozoites. These daughter merozoites are then released into the bloodstream where these merozoites invade the red blood cells. In *Plasmodium vivax* and *P. ovale* infections, the parasite in the hepatocytes may not mature into a schizont immediately. This remains dormant in the liver as hypnozoite and that makes it difficult to diagnose and treat. [Igweh 2012].

Within the red blood cell, the merozoite develops into a ring-shaped form, then to a trophozoite form and later into schizont and produce new daughter merozoites. The daughter merozoites are then released with the destruction of the red blood cell. Along with the new merozoites, toxic materials are released into the bloodstream that triggers the host immune responses that manifest the clinical signs of the disease. Some merozoites differentiate into male

and female gametocytes. The gametocytes are taken up by an *Anopheles* mosquito when it feeds on an infected individual. At the initial stages, the development of parasite occurs inside the blood bolus. The gametocytes are later liberated from the red blood cells and form the gametes. The gametes fuse to give rise to the zygote and then into the ookinete. The motile ookinete crosses the midgut epithelium into the hemocoel, where it stays between the epithelial surface and the basal lamina then transforms into an oocyst. The oocyst matures after 10-14 days and then releases sporozoites into the hemocoel. The sporozoites will travel to the salivary glands, stay within, and then get released each time the mosquito feeds [Abraham 2004] allowing the cycle of transmission to continue.

Infected red blood cells (iRBCs) of *P. falciparum* tend to form rosettes (Figure 2). Rosettes can cause vascular occlusion that contributes to the development of the severe complications of the disease such as cerebral malaria [Igweh 2012]. Cerebral malaria is one of the leading causes of death in young children despite rapid administration of chemotherapy [van der Heyde 2006]. The clinical features of human cerebral malaria (HCM) include neurological syndromes with patients in unrousable coma. Seizures, retinopathy, and brainstem alterations due to increased intracranial pressure and swelling of the brain are also observed. [Renia 2012]. Heparin, a highly sulfated glycosaminoglycan, can inhibit rosetting and cytoadherence of iRBCs *in vitro* [Xiao 1996]. It can also inhibit invasion of the erythrocytes by the *Plasmodium* merozoites [Wilson 2013]. It was at first used as adjunct therapy to malaria, but there was intracranial bleeding with heparin use and thus its use in clinical malaria has been discontinued [Igweh 2012].

Another complication of *P. falciparum* is placental malaria. Placental malaria is prevalent in primigravidae women especially in endemic countries. The effects on the mother and the fetus would be abortion, stillbirth, premature delivery, and low birth weight of the infant. In less severe cases, morbidity is presented by anemia and low birth weight of the child. This is characterized by the presence of parasites in intervillous spaces along with leukocytes, adhering to chondroitin sulfate A (CSA), a glycosaminoglycan receptor present in the placenta [Achur 2000; Matteelli 1997].

Current antimalarial therapies target the blood stage intraerythrocytic *Plasmodium* parasite [Wilson, 2013]. *P. falciparum* gametocytes are relatively insensitive to most antimalarials, except artemisinins and primaquine. That is why artemisinin combination therapies (ACTs) have the advantage of reducing both sexual and asexual stages of *P. falciparum*. Primaquine can effectively kill gametocytes thereby preventing transmission. However, primaquine also causes methemoglobinemia and hemolysis, which is disadvantageous to the malaria patient [Barnes 2012].

Parasite resistance to antimalarial drugs is a major hurdle in malaria control and elimination. Resistance to antimalarials are associated with return of illnesss, anemia, increased gametocyte carriage, which could promote the transmission of resistant parasites. Widespread resistance has been reported for chloroquine and sulfadoxine-pyrimethamine monotherapies. Recently, reported resistance to artemisinin, as shown by prolonged parasite clearance times even with adequate or increased drug exposure, along the Thai-Cambodian border alarmed the malaria control community [Barnes 2012; Grellier 2012]

With concerted efforts towards eliminating malaria which include greater distribution of insecticide-treated mosquito nets, increased use of residual spraying, use of rapid diagnostic tests services for early diagnosis and treatment of the disease with ACTs, there has been a decline in the morbidities and mortalities in sub-Saharan Africa [Kweka, 2013]. Other endeavors are dedicated to researches on elucidating molecular mechanisms for developing new drugs and vaccines.

There is growing interest in compounds from novel sources, such as seaweeds, and marine invertebrates as potential drugs. Sulfated polysaccharides derived from seaweeds such as fucoidan [Chen 2009], and carrageenans (Figure 3A, 3B, and 3C) [Adams, 2005], and diterpenes [Stout 2010] from algae have been assessed for their antimalarial effects. Fucoidan [Chen 2009] and the 3 types of carrageenan (namely, λ, ι, and κ) [Adams 2005] inhibit the *in vitro* growth and invasion of red blood cells by *P. falciparum*. Although the underlying mechanism how these sulfated polysaccharides work is still unclear, it may be similar to the action of heparin. Heparin and heparan sulfate interact with merozoite proteins [Zhang 2013; Kobayashi 2013] such as the merozoite surface protein 1 [Boyle 2010] and EBA-140 [Kobayashi 2010].

κ-carrageenan has been modified by acetylation, sulfation, and phosphorylation [Yuan 2005]. These κ-carrageenan derivatives were found to be active against tumor cells [Yuan 2006; Yuan 2011], bacteria [Wang 2011] and viruses, like influenza virus [Wang 2011; Tang 2013], and human immunodeficiency virus [Vlieghe 2002].

Gellan gum is a microbial polysaccharide produced by the bacterium *Sphingomonas* (*Pseudomonas*) *elodea* (ATCC 31461). It is a thermoreversible gel, that is currently utilized as drug vehicle, food additive, component of personal care products, and microbiological media. Its

basic repeating unit is a tetrasaccharide, consisting of two glucose (Glc) residues, one glucuronic acid (GlcA), and one rhamnose (Rha) residue: [→3)-β-D-Gl*cp*-(1→4)-β-D-Gl*cp*A-(1→4)-β-D-Gl*cp*-(1→4)-α-L-Rhap-(1→] [Ogaji 2012; Rinaudo 2000; Shah 2007; Goncalves 2009]. Gellan gum has been reported to be safe for internal use [Shah 2007]. Gellan sulfate is the sulfated derivative of gellan gum [Miyamoto 2001] that possesses anticoagulant activities similar to heparin. These novel sulfated polysaccharides can make interesting antimalarial candidates.

Figure legends

Figure 1. The life cycle of the *Plasmodium falciparum* parasite. *Plasmodium falciparum*, is transmitted during the blood meal of an infected female *Anopheles* mosquito. *P. falciparum* sporozoites in the saliva of the mosquito are injected into the dermis and migrate to the liver where they invade hepatocytes and produce daughter merozoites. The daughter merozoites are released into the bloodstream where they invade the red blood cells. Within the red blood cell, the merozoite undergoes cell division and develops into a ring, trophozoite and schizont forms, finally producing new daughter merozoites. The daughter merozoites are released with the destruction of the red blood cell. Some merozoites differentiate into male and female gametocytes that are taken up by an *Anopheles* mosquito when it feeds on the infected individual. Inside the mosquito gut, the gametocytes are free from the red blood cells and form the gametes. The gametes fuse giving rise to the zygote and then into the ookinete. The motile ookinete crosses the midgut epithelium into the hemocoel, where it stays between the epithelial surface and the basal lamina then transforms into an oocyst. The oocyst matures after 10-14 days and then releases sporozoites into the hemocoel. The sporozoites will travel to the salivary glands, stay within, and then get released each time the mosquito feeds allowing the cycle of transmission to continue.

Figure 2. Rosetting, sequestration and cytoadhesion of *Plasmodium falciparum* infected red blood cells. Rosetting, sequestration and cytoadherence to endothelial cells can cause vascular occlusion that contribute to the development of the severe complications of the disease such as cerebral malaria . This phenomenon is associated with parasite proteins such as PfEMP1 that are expressed in the iRBC membrane that interact with receptors including CD36, thrombospondin,

and ICAM1 on the endothelial cells and leukocytes.

Figure 3. Structures of carrageenans. **(a)** The structure of κ-carrageenan shows 1 sulfate group. **(b)** ι-carrageenan shows 2 sulfate groups. **(c)** λ-carrageenan shows 3 sulfate groups.

Fig. 1

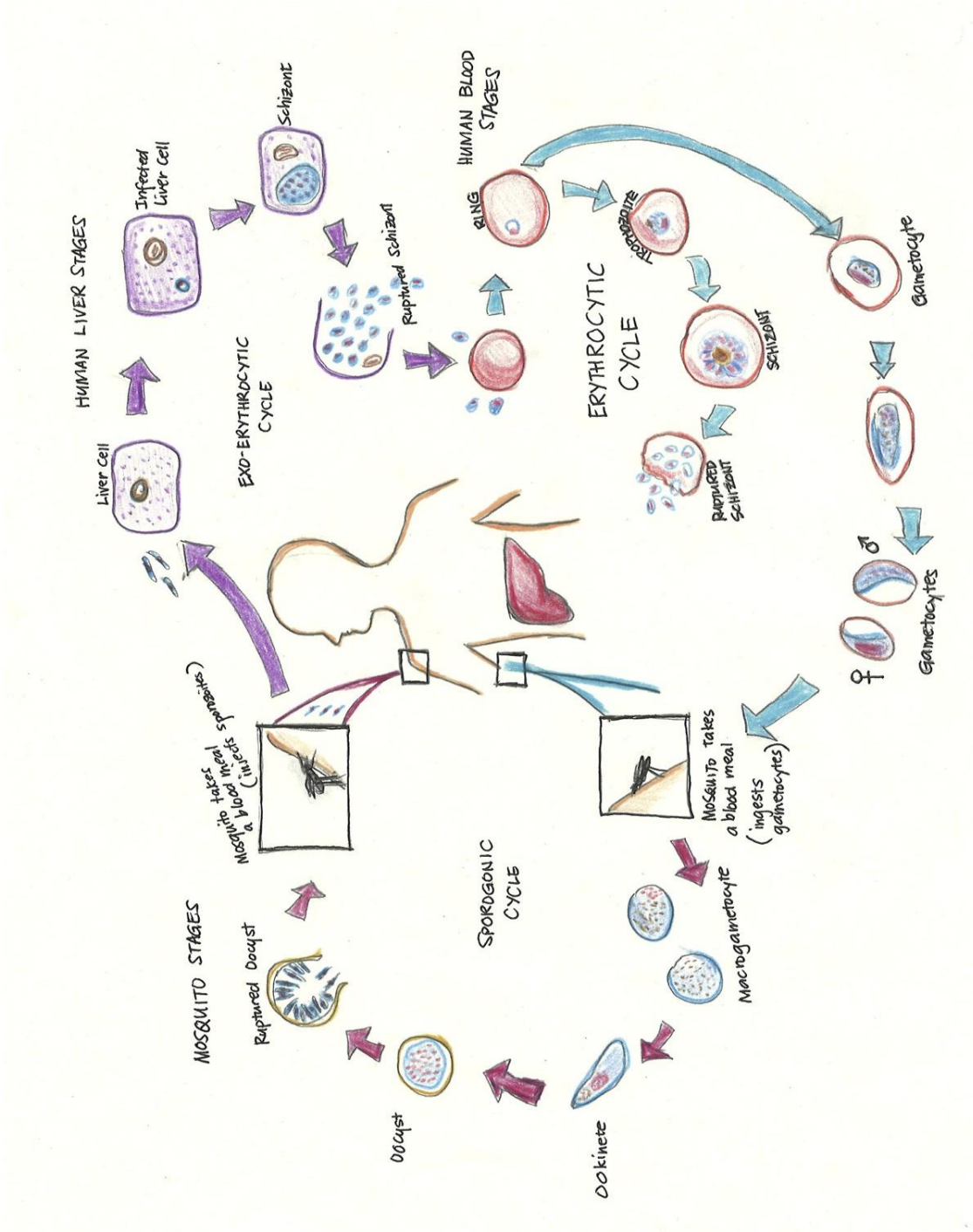
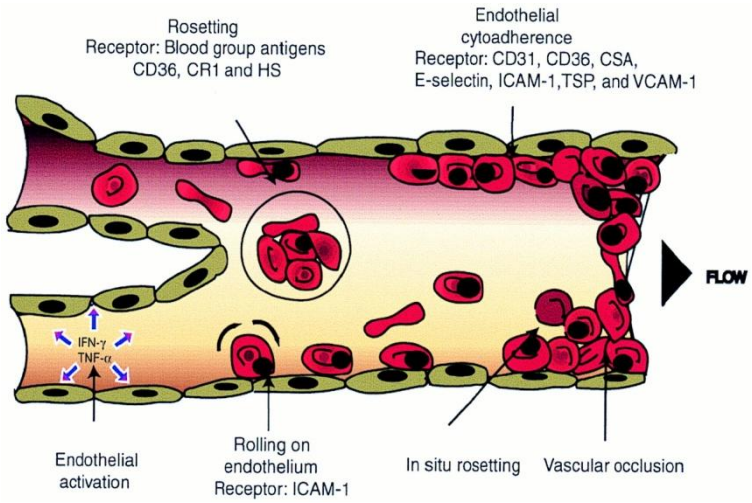
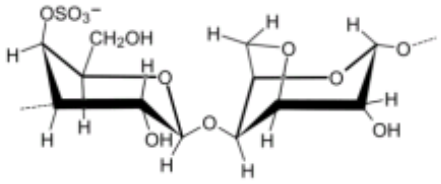

HUMAN LIVER STAGES
Liver Cell
Infected Liver Cell
Schizont
EXO-ERYTHROCYTIC CYCLE
Ruptured Schizont
HUMAN BLOOD STAGES
RING
ERYTHROCYTIC CYCLE
TROPHOZOITE
SCHIZONT
RUPTURED SCHIZONT
GAMETOCYTE
♂
♀
GAMETOCYTES
MOSQUITO STAGES
Mosquito takes a blood meal (injects sporozoites)
Ruptured Oocyst
Oocyst
Ookinete
SPOROGONIC CYCLE
Macrogametocyte
Mosquito takes a blood meal (ingests gametocytes)

Fig. 2

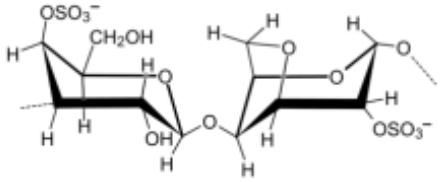


Chen Q *et al.* Clin. Microbiol. Rev. 2000;13:439-450

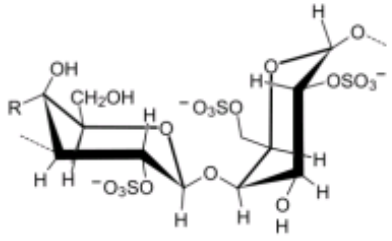
Fig. 3



κ−carrageenan



ι−carrageenan



λ−carrageenan

Necas, J., and Bartosikova, L.. 2013.

CHAPTER 1

Synthesis of gellan sulfate, oversulfated κ-carrageenans and hydrolyzed λ carrageenan and assessment of these polysaccharide derivatives on *Plasmodium falciparum* growth and invasion of red blood cells *in vitro*

Submitted to Scientific Reports

Abstract

This work shows the preparation and assessment of the sulfated derivative of the microbial polysaccharide gellan gum and derivatives of λ and κ-carrageenans for their *in vitro* inhibition of growth and invasion of red blood cells of *P. falciparum* 3D7 and Dd2. Gellan gum and κ-carrageenan were sulfated using DMF-SO_3, while λ –carrageenan was treated with HCl. The products were purified by dialysis and were added to parasite cultures. After 96 h, the cultures were assessed by flow cytometry for growth inhibition; and by counting the ring parasites after 20 h for invasion inhibition. Gellan sulfate showed strong inhibition of invasion and modest inhibition of growth for both *P. falciparum* 3D7 and Dd2 better than the native gellan gum. The hydrolyzed λ-carrageenan and oversulfated κ-carrageenan were less inhibitory than their native forms. *In vitro* cytotoxicity assays using the tetrazolium dye, MTT, showed that the modified polysaccharides were not toxic to the 293T cells *in vitro*. *In vitro* APTT anticoagulation assays showed that the synthesized gellan sulfate, in particular had low anticoagulant activity.

1. Introduction

There were an estimated 660,000 deaths from 220 million cases of malaria in 2010 [World Malaria Report, 2012]. This represents a decline in the mortality caused by malaria especially in the sub-Saharan endemic areas [Kweka, et al. 2013] and this success can be attributed to programs that include distribution of insecticide-treated nets, use of indoor residual spraying, and expansion of malarial rapid diagnostic tests (RDTs). But with the constant threat of the *Plasmodium* parasites and the *Anopheles* vectors developing resistance to established antimalarials and insecticides, the thrust to develop alternative antimalarial drugs, insecticides, and improved rapid diagnostic tests continues [Kweka, 2013].

Plasmodium parasites,are obligate intracellular protozoa transmitted from a blood meal of the female *Anopheles* mosquito to humans. In the erythrocytic stage of the disease, the *Plasmodium* merozoites invade red blood cells that results into the destruction of red blood cells and release of parasite and erythrocytic material into the circulation. The host response to these events is manifested into the clinical symptoms of the disease which include intermittent fever, abdominal pain, anemia and overall weakness [Sherman, 1998].

Successful invasion of the erythrocyte is crucial for the survival of the malaria parasite. Upon egress, there is a short time window that the free malaria merozoites are exposed to the host's immune system, which also provides an opportunity to target the parasites with vaccines or drugs [Yahata 2012].

Erythrocyte invasion by the *Plasmodium* merozoite is a complex, multi-step process that involves interactions between the parasite and host cell proteins. The initial reversible attachment of the merozoite to the red blood cell may involve proteins on the merozoite surface, although

evidence to support this concept is needed. In the committed red cell binding, the merozoite reorients so that the apical complex makes contact with the erythrocyte surface. Here, the micronemes secrete invasion proteins, such as apical membrane antigen 1 (AMA1) and erythrocyte binding-like proteins (EBLs), and the rhoptries secrete reticulocyte binding-like proteins (RBLs) and rhoptry neck proteins (RONs). AMA1, a major candidate for a multicomponent vaccine against malaria [Schwartz 2012], interacts with the rhoptry neck proteins RON2, RON4, and RON5 to form a complex that is a critical component of the moving junction. The EBLs and RBLs bind with receptors on the red blood cell and are implicated in host cell selection and alternative invasion pathways for the *Plasmodium* merozoite. Merozoite entry into the red blood cell proceeds as the merozoite is propelled by an actin-myosin motor complex, with simultaneous shedding of the surface proteins to enable the parasite to adapt to its new intracellular environment [Cowman 2012, Gaur 2011, Harvey 2012].

Sulfated glycosaminoglycans (GAGs), such as heparin [Kulane 1992], dextran sulfate, fucoidan [Clark 1997, Xiao 1996], and carrageenans [Adams 2005] have been shown to inhibit merozoite entry into erythrocytes and prevent the cytoadherence of *Plasmodium*-infected red blood cells *in vitro* [Clark 1997, Xiao 1996].

Heparin, in particular, has been shown to target several merozoite proteins [Zhang, 2013, Kobayashi, 2013] including invasion proteins such as merozoite surface protein 1 (MSP1) [Boyle, 2010] and erythrocyte binding antigen 140 (EBA-140) [Kobayashi 2010]. Thus, unlike other antimalarials, heparin can inhibit the invasion of red blood cells by the *Plasmodium* merozoites [Wilson, 2013]. MSP1 is a major candidate for a multi-component malaria vaccine [Schwartz 2012], and EBA-140, also known as BAEBL, is an erythrocyte binding ligand that interacts with the glycophorin C receptor on the erythrocyte surface [Lobo, 2003]. These

findings provide clues on how these invasion proteins interact with cell surface proteoglycans, such as heparan sulfate, and could help explain the mechanism by which sulfated polysaccharides inhibit parasite entry into red blood cells. However, because heparin is a potent anticoagulant, it cannot be used to treat clinical malaria [WHO, 2010].

Sulfated polysaccharides from marine sources are currently being exploited for their potential therapeutic applications. These include carrageenans from seaweeds, of which there are three major types, namely kappa (κ), lambda (λ), and iota (ι). These carrageenans differ in their levels of sulfation, which may also account for their different gelling properties. The carrageenans have been previously shown to inhibit the growth and invasion of red blood cells by *Plasmodium falciparum* 3D7 and Dd2 *in vitro* [Adams 2005]. *In vivo,* A/J mice pre-treated with calcium carrageenan prior to infection with *P. berghei* NK65A showed lower parasitemia compared with the untreated group, and a slight delay in the deaths of the infected mice [James 1981]. In addition, Huber *et al.* [Huber 2002] showed that there was an increase in the permeability of the blood brain barrier in rats 72 h after subcutaneous administration of λ-carrageenan. This side effect could enhance the development of cerebral malaria and is thus a major disadvantage of carrageenans for use in malaria.

Studies on κ-carrageenan derivatives have shown improved potential of these derivatives against various pathogens. Acetylated, sulfated, and phosphorylated κ-carrageenan derivatives were assessed for their antioxidant and antitumor properties [Yuan, 2005]. Other κ-carrageenan derivatives were found to be active against tumor cells [Yuan 2006, Yuan 2011], bacteria [Wang 2011] and viruses, including influenza virus [Wang, 2011, Tang 2013], and human immunodeficiency virus [Vlieghe 2002]. I wanted to determine how modification of κ-

carrageenan and λ-carrageenan could affect their action against malaria parasites and their safety as potential adjunct therapy for malaria.

Gellan gum is a linear, anionic, high molecular weight, microbial polysaccharide produced by the bacterium *Sphingomonas (Pseudomonas) elodea* (ATCC 31461). It is a thermoreversible gel, noted for its high gel strength and stability that make it useful as a drug vehicle, food additive, component of personal care products, and microbiological media. Its basic repeating unit is a tetrasaccharide, consisting of two glucose (Glc) residues, one glucuronic acid (GlcA), and one rhamnose (Rha) residue: [→3)-β-D-Gl*cp*-(1→4)-β-D-Gl*cp*A-(1→4)-β-D-Gl*cp*-(1→4)-α-L-Rhap-(1→] [Ogaji 2012, Rinaudo 2000, Shah 2007, Goncalves 2009]. Tests on animals in which gellan gum was added to the diet or given by gavage, have shown that it is inert and safe for internal use [Shah 2007].

Gellan gum has been modified to obtain sulfated derivatives (gellan sulfate) [Miyamoto 2001] that were shown to have anticoagulant activities similar to heparin, except for one derivative, GS1, which had the lowest sulfonation ratio (5.0%), and showed low anticoagulant activity [Miyamoto 2010]. At present, there are no published reports on the *in vitro* effect of gellan gum and gellan sulfate on malaria parasites.

I wanted to assess synthetic compounds that can inhibit merozoite entry into red blood cells, similarly to heparin but with a better safety profile. In the present study, I evaluated the microbial polysaccharide gellan gum and prepared its sulfated derivative, gellan sulfate, and the carrageenan derivatives, hydrolyzed λ-carrageenan and oversulfated κ-carrageenan, for their inhibitory effects on the growth and invasion of erythrocytes by *Plasmodium falciparum* 3D7

and Dd2. I also tested these compounds for their cytotoxicity to 293T cells *in vitro*. The *in vitro* anticoagulant activity of the synthesized gellan sulfate was also assessed.

2. Materials and Methods

2.1. Compounds and synthesis of gellan sulfate and carrageenan derivatives

The polysaccharides used in this study were heparin (Sigma), λ-carrageenan (Sigma), κ-carrageenan (WAKO), and gellan gum (WAKO). Hydrolyzed λ-carrageenan, oversulfated κ-carrageenan, and gellan sulfate were synthesized based on previously described methods with some modifications.

Lambda carrageenan was hydrolyzed with hydrochloric acid at 70°C [Yuan 2005]. The mixture was then evaporated and lyophilized, and the resulting powder was dialyzed against distilled water. Final lyophilization yielded a light brown powder. For the oversulfated κ-carrageenan [Yuan 2005] and gellan sulfate [Miyamoto 2001], sulfation was done by adding $HClSO_3$ in DMF at 60°C after acid hydrolysis with HCl at 70°C. After evaporation, lyophilization, and dialysis against distilled water, the resulting products were a brown powder of the oversulfated κ-carrageenan derivative and a white powder of gellan sulfate.

Prior to mixing with the culture assays, the polysaccharides were first dissolved in distilled water to a concentration of 2 mg/ml, and then filtered through a 0.22-μm filter and then further diluted with the appropriate complete media. Polysaccharides that were insoluble at room temperature, namely; κ-carrageenan, λ-carrageenan, and gellan gum, were dissolved in distilled water at 80°C, while heparin, oversulfated κ- carrageenan, hydrolyzed λ-carrageenan, and gellan sulfate were dissolved at room temperature.

2.2. Analytical methods

^{13}C NMR spectra were obtained from a JEOL 500 MHz spectrometer operating at 500 MHz. The spectra for the oversulfated κ-carrageenan were recorded at 25 °C, while the spectra for the native gellan gum and the gellan sulfate were recorded at 90°C. Chemical shifts (δ in ppm) were expressed relative to the resonance of D_2O (^1H NMR) (δ =4.8) and DMSO (^{13}C NMR) (δ =39.5). The samples for NMR analysis were dissolved in DMSO (oversulfated κ-carrageenan=60 mg/ml; native gellan gum=5 mg/ml; gellan sulfate 20 mg/ml) and trace amount of D_2O was added afterward.

The analysis of concentration of S was performed with Ion Chromatography System Dionex ICS-1100/1600 (Thermo Scientific Inc., MA USA). The analysis of concentraion of C was performed with 2400 Series II CHNS/O System (Perkin Elmer Inc., MA, USA). Degree of substitution (DS) was calculated using the formula given by Rochas *et al* [Rochas 1986]: [Eq. 1]

DS = (S %/mol. wt. S) / (mol. wt. C x mol. no. of C)

2.3. *In vitro* culture of *Plasmodium falciparum* parasites

The chloroquine-sensitive line *Plasmodium falciparum* 3D7 and the chloroquine-resistant line, *P. falciparum* Dd2 parasites were maintained in continuous culture according to the methods described by Radfar *et al* [Radfar 2006].In this method, the parasites are grown in human A^+ red blood cells and maintained in culture medium containing RPMI 1640, 25 mM HEPES, 100 μM hypoxanthine, 12.5 μg/ml gentamicin sulfate supplemented with 2.5% (w/v) Albumax II and 62.5 μg/ml of $NaHCO_3$. Cultures were kept at 37°C, 5% CO_2, and 5% O_2 with daily medium changes; conditions were maintained at 1% hematocrit and 1% parasitemia.

2.4. Cell culture

Human embryonic kidney derived 293T cells were grown at 37°C, 5% CO_2, and 5% O_2 in culture medium containing DMEM, L-glutamine, penicillin-streptomycin, and 62.5 µg/ml $NaHCO_3$, supplemented with 10% fetal calf serum and passaged every two days at 70%–80% confluency.

2.5. Growth Inhibition Assay

Growth inhibition assays (GIAs) were performed as previously described [Persson, 2006]. Predominantly ring stage cultures were first synchronized by sorbitol treatment. GIAs were carried out at the next day, when the cultures were mostly in the late trophozoite stage. Infected red blood cells were mixed with fresh Type A^+ RBCs to make cultures with 0.30% parasitemia and 1% hematocrit. A 25-µl aliquot of culture was then transferred into 96-well plates and 2.5 µl of the inhibitors was added to each well such that the final concentrations were 9, 4.5, 2.25, and 0.9 µg/ml. Three independent experiments were performed and all were done in triplicate and parallel cultures were also maintained. The cultures were incubated at 37°C, with 5% CO_2, and 5% O_2. At 48 h post-incubation, 5 µl of complete medium was added to each culture. Flow cytometry was used to determine the parasitemia after 90–96 h when the parasites were mostly in the trophozoite-schizont stages.

2.6. Flow cytometric analysis

Flow cytometric analysis was carried out to determine the parasitemia of the cultures [Persson, 2006]. Cultures were mixed with 10 μg/ml ethidium bromide in PBS and were left at room temperature for 1 h in the dark. The cell pellets were then resuspended in 500 μl of PBS and transferred into flow cytometry tubes. Flow cytometric analysis was carried out on a FACSCalibur (BD Biosciences) and the data were analyzed with WinMDi ver. 2.9.

2.7. Invasion Inhibition Assay

For the invasion inhibition assays [Bates 2010, Spadafora, 2011], cultures were purified by using MACS® (Miltenyl Biotec, Japan) magnetic beads separation column to obtain trophozoite and schizont stage parasites. Purified schizonts were mixed with complete medium to obtain hematocrit of 1%, and fresh A^+ red blood cells were added for a total parasitemia of 5% with ring stage parasitemia of almost 0%. Cultures (100 μl) were transferred into 96-well round bottom plates and the inhibitors heparin, gellan gum, and gellan sulfate were added to a final concentration of 100 μg/ml. As a negative control, distilled water was added to cultures to a final concentration of 5% and was incubated for 20 h at 37°C, with 5% CO_2 and 5% O_2. These assays were done in triplicate and at least three independent similar experiments were performed.

2.8. Assessment of invasion inhibition

Culture supernatants were aspirated and the cell pellets were smeared and stained with Giemsa staining to determine ring stage parasitemia. Means and standard deviations of ring stage parasitemia were obtained and the percent inhibition was computed using the following formula: [Eq.2] {(Mean % Ring stage parasitemia of DW-treated culture – Mean % Ring stage

parasitemia of inhibitor-treated culture) ÷ Mean % Ring stage parasitemia of DW-treated cultures} x 100% ; DW = distilled water.

2.9. Cytotoxicity assays

MTT assays were performed using 293T cells. The cells were seeded in 96-well flat-bottom wells with a culture volume of 100 µl at a 2:1000 dilution and incubated at 37°C, 5% CO_2, and 5% O_2. After 24 h, when the cells reached about 50% confluency, the old media were aspirated and replaced with 100 µl of complete medium containing inhibitors at concentrations of 500, 250, 100, 50, 25, and 10 µg/ml. These inhibitors were mixed with the cells and incubated at 37°C, 5% CO_2, and 5% O_2 for 48 h. MTT solution (10µl of 5mg/ml) was added to the culture, which was kept in the dark at room temperature overnight. The following morning, the old media were aspirated and 100 µl of DMSO was added as an MTT solvent. The cells were then assessed by measuring the absorbance at 595nm and the % cell viability was computed as follows [Edmondson, et al.1988]: [Eq. 3] % cell viability = (absorbance$_{595nm}$ of treated group ÷ absorbance$_{595nm}$ of control group) x 100 %.

2.10. *In vitro* Anticoagulant Activated Partial Thromboplastin Time (APTT) Assay

Blood was collected from the hearts of normal adult female C57BL/6 mice under terminal ether anesthesia. The bloods collected were mixed immediately with 3.2% trisodium citrate at 9:1 blood: anticoagulant ratio. The plasma were obtained by spinning the blood in a bench top centrifuge for 15 minutes and pooled. Ten µl of heparin, gellan gum and gellan sulfate each diluted in PBS were mixed with the 100 µl plasma for a final concentration of 10 µg/ml and then incubated at 37°C for 1 min [Shi, et al. 2007]. APTT assay was performed using

Drihemato® APTT Test Reagent Card (A&T Corporation, Japan) according to manufacturer's instructions. Fifty µl of plasma was added to a drop of Drihemato® PT-APTT Specimen Diluent B and mixed thoroughly. Twenty-five µl of the plasma and diluent mixture was then placed on the Drihemato® APTT Test Reagent Card and the coagulation time was read in a Drihemato® system Coag2V machine. The coagulation times were recorded in seconds (s).

2.11. Animals

Adult female C57BL/6 mice purchased from CLEA Japan (Tokyo, Japan) were maintained in controlled light and dark conditions and were given commercial feed and water ad libitum. Animal experiments were conducted in accordance with the Principles for the Care and Use of Research Animals of Obihiro University of Agriculture and Veterinary Medicine. The protocol for the animal experiments was approved by the Committee on the Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit No. 25-153, 2013).

3. Results

3.1. Synthesis of gellan gum, λ- and κ-carrageenan derivatives

κ-Carrageenan and gellan gum were sulfated by SO_3-pyridine complex. The κ-carrageenan and gellan sulfates, and the hydrolyzed λ-carrageenan were more readily soluble in distilled water at room temperature compared with their native counterparts. The modified polysaccharides also did not form gels in solution.

Degree of substitution (DS) of the sulfonyl group and sulfation rate (%) were determined by elemental analyses, as listed in Table 1. DSs for κ-carrageenan and gellan were defined as molar number of sulfonyl group per di-saccharide and tetra-saccharide units, respectively

NMR analyses were employed to confirm sulfation of κ-carrageenan and gellan. ^{13}C-NMR spectra before and after sulfation are given in Figures 1 and 2. The peaks of native κ-carrageenan and gellan were assigned according to the assignments reported in the previous articles [Jay 1998, van de Velde 2002, de Araujo 2013]. The major peaks of sulfated κ-carrageenan with DS of 3.0 were also assigned according to the assignments for sulfated κ-carrageenan with DS of 3.1 reported in the previous studies [de Araujo 2013, Thahn 2001]. The overlapped peaks of ring-carbons of gellan sulfate with DS of 3.7 were hardly assigned due to their complex appearance caused by sulfonyl substituents and remaining hydroxyl groups. In the case of κ-carrageenan, shift of the peaks of C6 (G) from 61.4 to 66.5 ppm, C2 (G) from 69.7 to 75.4 ppm and C2 (A) from 70.0 to 72.7 ppm are indicative for successful sulfation at these positions. Complete disappearance of the peak assigned C6 (G) is indicating that C6 (G) position is completely substituted. Other peaks are likely assigned to ring carbons of un-substituted sugar units. In the case of gellan, the peaks assigned to C6 (13G) at 61.7 ppm, C6 (14G) at 62.2 ppm, C2 (R) at 71.6 ppm, and C3 (R) at 71.7 ppm, completely disappeared,

indicating preferential sulfation at these positions.

3.2. Growth Inhibition Assay

The native λ- and κ–carrageenans inhibited the growth of *Plasmodium falciparum* 3D7 (Figure 3A) and Dd2 (Figure 3B) *in vitro* consistent with the observations of Adams *et al* [Adams 2005] in a concentration-dependent manner. Both the hydrolyzed λ-carrageenan and the oversulfated κ-carrageenan derivatives showed poor inhibition of *P. falciparum* 3D7 and Dd2 growth *in vitro*. Gellan sulfate (9 μg/ml) inhibited growth of *Plasmodium falciparum* 3D7 (55.33%) and Dd2 (85.83%) *in vitro* (Figure 3A and 3B). In contrast, native gellan gum exhibited low inhibition (23.04%, 3D7; and, 39.22%, Dd2) at the same concentration. From here, I chose to further assess gellan sulfate for its ability to inhibit invasion.

3.3. Invasion Inhibition Assay

At 100 μg/ml, gellan sulfate showed strong inhibition of invasion of *Plasmodium falciparum* 3D7 (96.15%) (Figure 4A and 4C) and Dd2 (98.17%) (Figure 4B and 4D) *in vitro* that was comparable to that of heparin (98.79% for 3D7 and 96.16% for Dd2). Native gellan gum, on the other hand, showed little inhibition of invasion for both *Plasmodium falciparum* 3D7 and Dd2.

3.4. Cytotoxicity Assays

Unlike the native gellan gum, gellan sulfate does not gel in solution. With this change in property I wanted to determine the safety of the gellan sulfate for future *in vivo* studies. Hence, I performed MTT cytotoxicity assays on 293T cells using the original selection of inhibitors for comparison (Figure 5A). Heparin and native gellan gum treatment caused a marked decrease in cell viability at concentrations of 250 and 500 μg/ml (54.68% and 70.18% for heparin; 79.86% and 57.74% for gellan gum). By contrast, gellan sulfate, as well as the native λ- and κ-carrageenans and their respective derivatives, showed relatively low cytotoxicity at 500 μg/ml (86.44%, 81.52%, 89.33%, 101.88% and 94.30%, respectively).

3.5. *In vitro* anticoagulant activity of gellan sulfate

The *in vitro* anticoagulant activity of the gellan sulfate was assessed by activated partial thromboplastin time (APTT) anticoagulant assays using healthy mouse plasma [Shih 2007]. In separate experiments, the normal APTT value for plasma from a healthy animal ranged from 21.8 s to 27.1 s, with mean value of 23.4 s. As shown in Figure 5B, the APTT for plasma with heparin at 10 μg/ml increased dramatically (mean = 181.0 s), whereas for plasma with 10 μg/ml gellan sulfate, the mean APTT was 28.7 s. The plasma with 10μg/ml native gellan gum had a mean APTT time of 22.5 s. This shows that the synthesized gellan sulfate has low anticoagulant activity.

4. Discussion

Polysaccharides from marine sources are currently gaining popularity for their potential medical and therapeutic applications. Seaweed-derived polysaccharides like carrageenans [Adams 2005] and fucoidan [Chen 2009] have been shown to inhibit the growth and invasion of *P. falciparum in vitro*. However, in *in vivo* studies, although a decrease in parasitemia may have been observed, there was no significant improvement in the recovery of mice from the illness [James 1981, Chen 2009]. In addition, λ-carrageenan was found to increase the permeability of the blood-brain barrier of rats when injected subcutaneously [Huber 2002]. This may aggravate the disease by adding to the development of cerebral malaria.

Although the exact mechanism of inhibition by these sulfated polysaccharides is yet to be defined, it may be related to the interactions of the malarial invasion proteins with the sulfated proteoglycans like heparan sulfate and chondroitin sulfate that are present on the surface of most cell types including the erythrocyte. These interactions have been demonstrated by Boyle *et al*. [Boyle 2010] and our group [Kobayashi 2013], where heparin was shown to interact with the invasion proteins MSP1 and EBA-140, respectively.

Naturally-occuring polysaccharides have been modified by sulfation and assessed for their biological activities such as anticoagulant, antibacterial and antiviral activities. Galactomannans from seed extracts that were modified by sulfation have been shown to have activities against yellow fever virus and dengue 1 virus *in vitro* and *in vivo* [Ono, et al, 2003], and against HIV *in vitro* [Muschin 2012]. Sulfated konjac glucomannans, have also been shown to act against HIV [Bo 2013].

Modification of κ-carrageenan has been shown to improve its action against a variety of pathogens. Low molecular weight κ-carrageenan (3 kDa) and κ-carrageenan oligosaccharides

(KOS), as well as their sulfated derivatives have been shown to have anti-influenza virus effects both *in vitro* and *in vivo* [Wang 2011, Tang 2013]. KOS was also reported to have enhanced immunostimulatory and antitumor activities [Yuan 2005, Yuan 2006]. In addition, κ-carrageenan that was covalently bound to 3'-azido-3'-deoxythymidine AZT, was found to be active against human immunodeficiency virus *in vitro*.

Gellan sulfate has been prepared and intended for various medical applications such as an artificial ligand to remove the extra domain A containing fibronectin (EDA(+)FN) from plasma of rheumatoid arthritis (RA) patients [Miyamoto 2001], and as a novel anticoagulant [Miyamoto 2010].

Thus, I synthesized oversulfated κ-carrageenan and gellan sulfate and I assessed the activities of these derivatives against malaria parasites *in vitro*. Elemental analysis showed that the synthesized oversulfated κ-carrageenan and gellan sulfate have sulfate content of 13.6% and 9.57%, respectively (Table 1.). The degree of substitution values (DS) were calculated as 3.0 for κ-carrageenan and 3.7 for gellan [Rochas 1986, Melo 2002]. Note that maximum DSs for carrageenan and gellan are 4 and 10, respectively. Sulfation rates (%) of κ-carrageenan and gellan sulfates relative to maximum DS were calculated as 75 % and 37 %.

The synthesized oversulfated κ-carrageenan and hydrolyzed λ-carrageenan did not show any activity against *Plasmodium* parasites which is different from the previously reported modified carrageenans. These differences could be attributed to the different modes of invasion and replication of the viruses within host cells compared with that of parasites.

The results show that the synthesized gellan sulfate effectively inhibited both the growth and invasion of the *P. falciparum* merozoites *in vitro* unlike the native gellan gum yet similar to

heparin. However, unlike heparin, gellan sulfate makes a suitable selective artificial ligand for EDA(+)FN from plasma of rheumatoid arthritis (RA) patients because it does not bind to proteins such as plasma fibronectin and antithrombin III [Miyamoto 2001]. Gellan sulfates with 17.5% sulfation or greater were also shown to be potent anticoagulants like heparin [Miyamoto 2010]. However, based on the cytotoxicity and anticoagulant assays the synthesized gellan gum with 37% sulfation was shown to have low anticoagulant activity. The reason for this difference, though, is unclear at this point.

Figure legends

Figure 1. ^{13}C NMR spectra of (a) unsubstituted κ-carrageenan and (b) oversulfated κ-carrageenan (R = SO3- or H). G-S and A-S represent sulfated G and A units. Shift of the peaks of C6 (G) from 61.4 to 66.5 ppm, C2 (G) from 69.7 to 75.4 ppm and C2 (A) from 70.0 to 72.7 ppm are indicative for successful sulfation at these positions. Complete disappearance of the peak assigned C6 (G) is indicating that C6 (G) position is completely substituted.

Figure 2. ^{13}C-NMR spectra of (a) gellan (R = H) and (b) sulfated gellan (R = SO_3- or H). The complete disappearance of peaks assigned to C6 (13G) at 61.7 ppm, C6 (14G) at 62.2 ppm, C2 (R) at 71.6 ppm, and C3 (R) at 71.7 ppm, indicate preferential sulfation at these positions.

Figure 3. *In vitro* growth inhibition assay of *P. falciparum* 3D7 (A) and Dd2 (B). Late stage *P. falciparum* 3D7 and Dd2 trophozoites (parasitemia 0.3 %, hematocrit 1%) were cultured in the presence of the following inhibitors: heparin (HEP), gellan gum (GG), gellan sulfate (SGG), λ-carrageenan (LA), hydrolyzed λ-carrageenan (HLA), κ-carrageenan (KP), and oversulfated κ-carrageenan (OSK) in 96-well round-bottom plates for 96 h. Final parasitemia was determined by use of flow cytometry. Percent inhibition is shown (y-axis) against increasing concentrations (x-axis) of each inhibitor. At 9μg/ml, SGG inhibited the growth of the *P. falciparum* 3D7 by 56% and Dd2 by 80%. At the same concentration, GG, HLA, and OSK inhibited both *P. falciparum* clones by 20% or less. In three independent assays, SGG consistently inhibited the growth of *P. falciparum* 3D7 by less than 60% and Dd2 by 70-80% (data not shown).

Figure 4. *In vitro* invasion inhibition assay of *P. falciparum* 3D7 and Dd2. MACS-purified *P. falciparum* 3D7 and Dd2 schizonts were cultured in 96-well round-bottom plates (ring stage parasitemia = 0%, hematocrit = 1%) for 20 h in the presence of 100 μg/ml heparin (HEP 100), gellan gum (GG 100), gellan sulfate (SGG 100), or distilled water (DW). Percent inhibition of invasion by *P. falciparum* 3D7 and Dd2 is shown in (A) and (C), respectively. Ring stage parasitemia for each treatment, as determined by examining Giemsa-stained smears, is shown in (B) for *P. falciparum* 3D7 and in (D) for *P. falciparum* Dd2. Untreated groups are labeled as (Untreated). All assays were done in triplicate wells and three independent experiments were performed.

Figure 5. Cytotoxicity and Activated Partial Thromboplastin Time (APTT) assays (a) Cytotoxicity assays using 293T cells. Cultures of 293T cells (100 μl) were seeded into 96-well flat-bottom plates and incubated at 37°C. At about 50% confluence, the old media were replaced with fresh media containing the inhibitors heparin (HEP), gellan gum (GG), gellan sulfate (SGG), λ-carrageenan (LA), hydrolyzed λ-carrageenan (HLA), κ-carrageenan (KP), or oversulfated κ-carrageenan (OSK) and the cells were incubated at 37°C for 48 h. MTT (10 μl) was then added to the cultures, which were kept in the dark overnight (about 12 h) at room temperature. Absorbance was read at 595 nm and the % cell viability was computed as: % cell viability = (absorbance$_{595nm}$ of treated group ÷ absorbance$_{595nm}$ of control group) x 100%. Means and standard deviations from the means were computed from triplicates assay in three independent experiments. **(b) Activated Partial Thromboplastin Time (APTT) Assays.** Pooled plasma from adult C57BL/6 mice was used for the APTT anticoagulant assays. Ten μl of heparin, gellan gum and gellan sulfate each diluted in PBS were mixed with the 100 μl plasma

for a final concentration of 10μg/ml and then incubated at 37°C for 1 minute. APTT assay was performed using Drihemato® APTT Test Reagent Card (A&T Corporation, Japan) according to manufacturer's instructions. Fifty μl of plasma was added to a drop of Drihemato® PT-APTT Specimen Diluent B and mixed thoroughly. Twenty-five μl of the plasma and diluent mixture was placed on the Drihemato® APTT Test Reagent Card and the coagulation time (in seconds) was read in a Drihemato® system Coag2V machine. Two independent assays were performed in triplicates and means and standard deviations were calculated.

CHAPTER 1

Table 1. Characteristics of sulfated κ-carrageenan and gellan gum

Table 1. Characteristics of sulfated κ-carrageenan and gellan gum

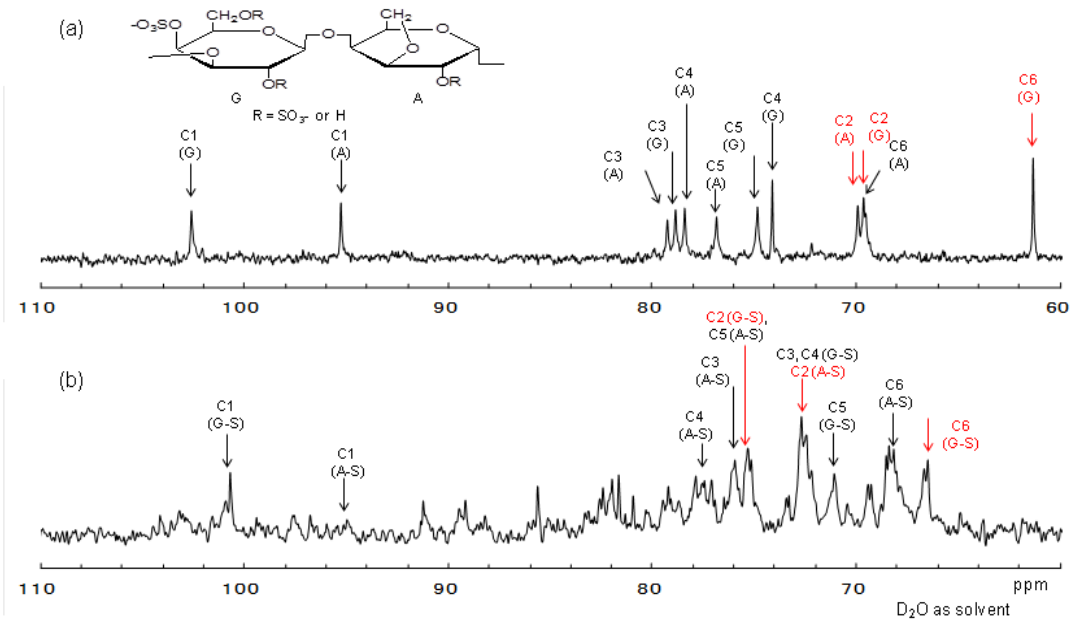
Sulfated derivatives	Elemental analysis (%)			DS[a]	Sulfation rate (%)[a]
	C	H	S		
Carrageenan	20.3	4.09	13.6	3.0	75
Gellan	23.3	4.38	9.57	3.7	37

[a]Relative to maximun DS: 4.0 (κ-carrageenan), 10.0 (gellan)

Percent carbon (C), hydrogen (H), and sulfur (S) as determined by elemental analyses. DS was calculated according to the equation given by Rochas *et al* [Rochas 1986] as follows. DS=(S%/atomic mass of S)/(C%/atomic mass of C × number of carbons for one unit). Sulfation rate (%) was calculated by taking ratio of DS of sulfonyl group to maximum DS of 4 for κ-carrageenan and 10 for gellan, respectively

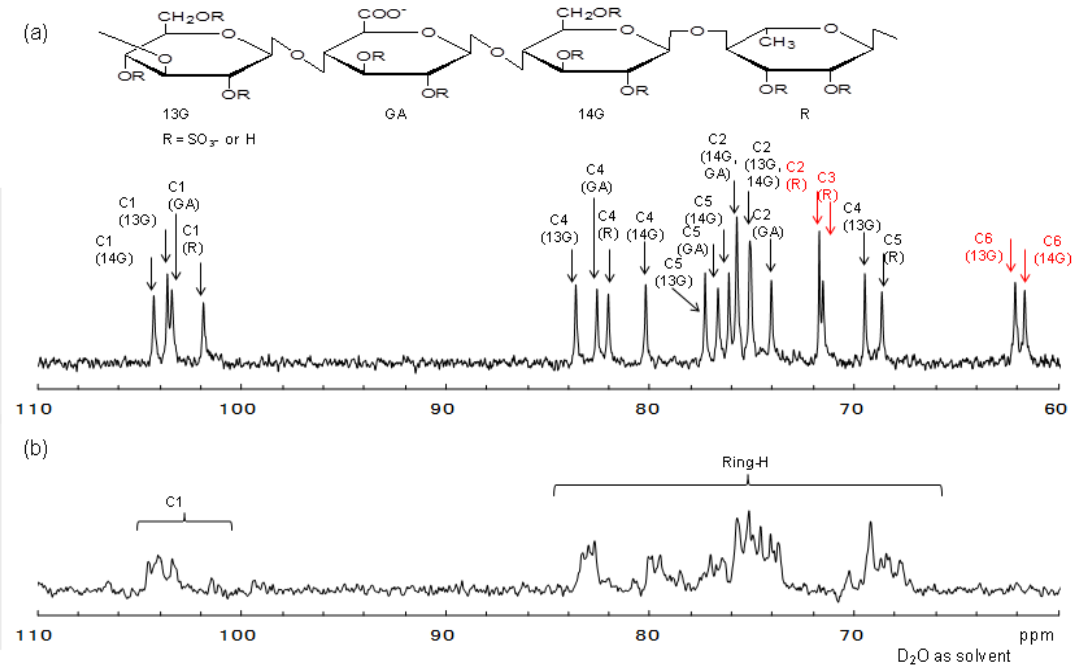
CHAPTER 1

Fig. 1



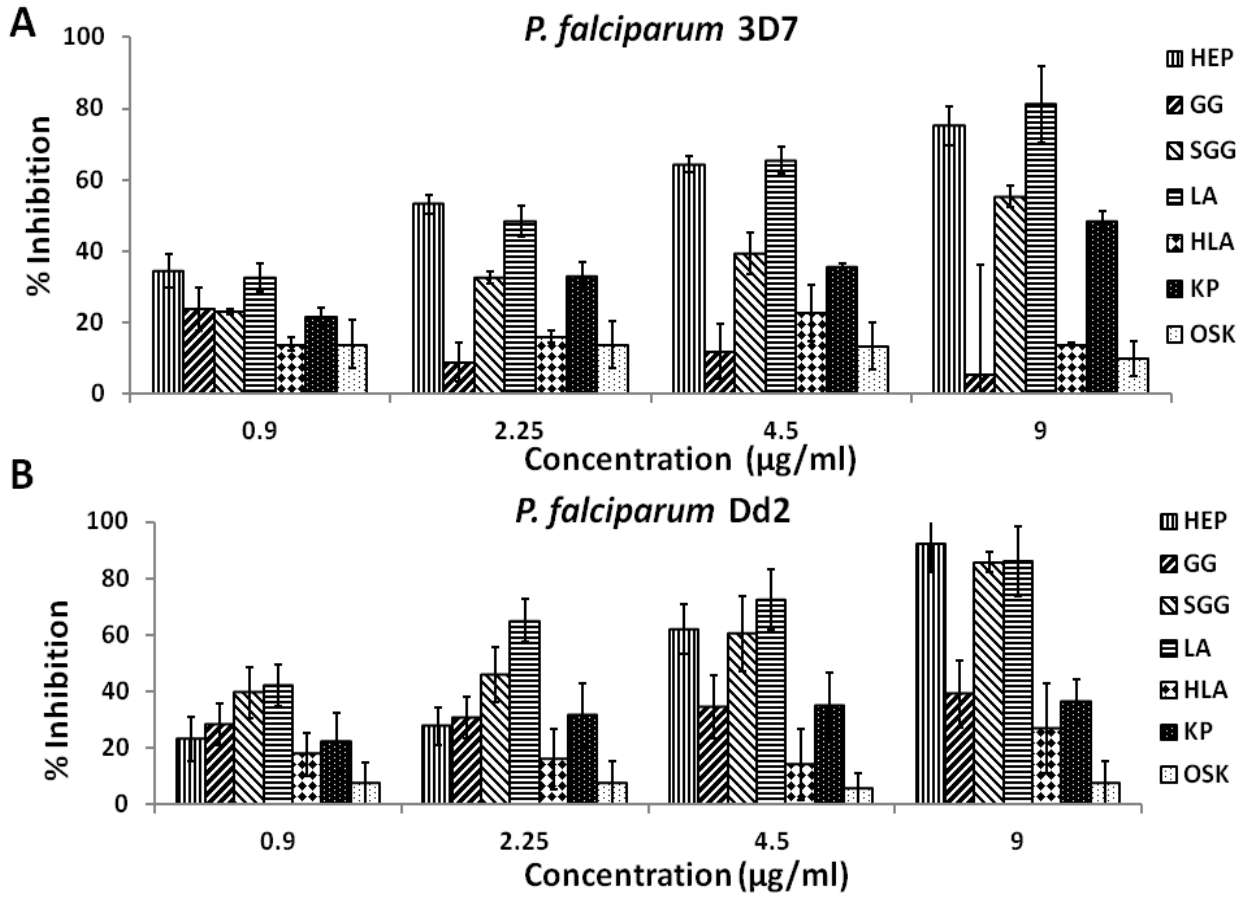
CHAPTER 1

Fig. 2



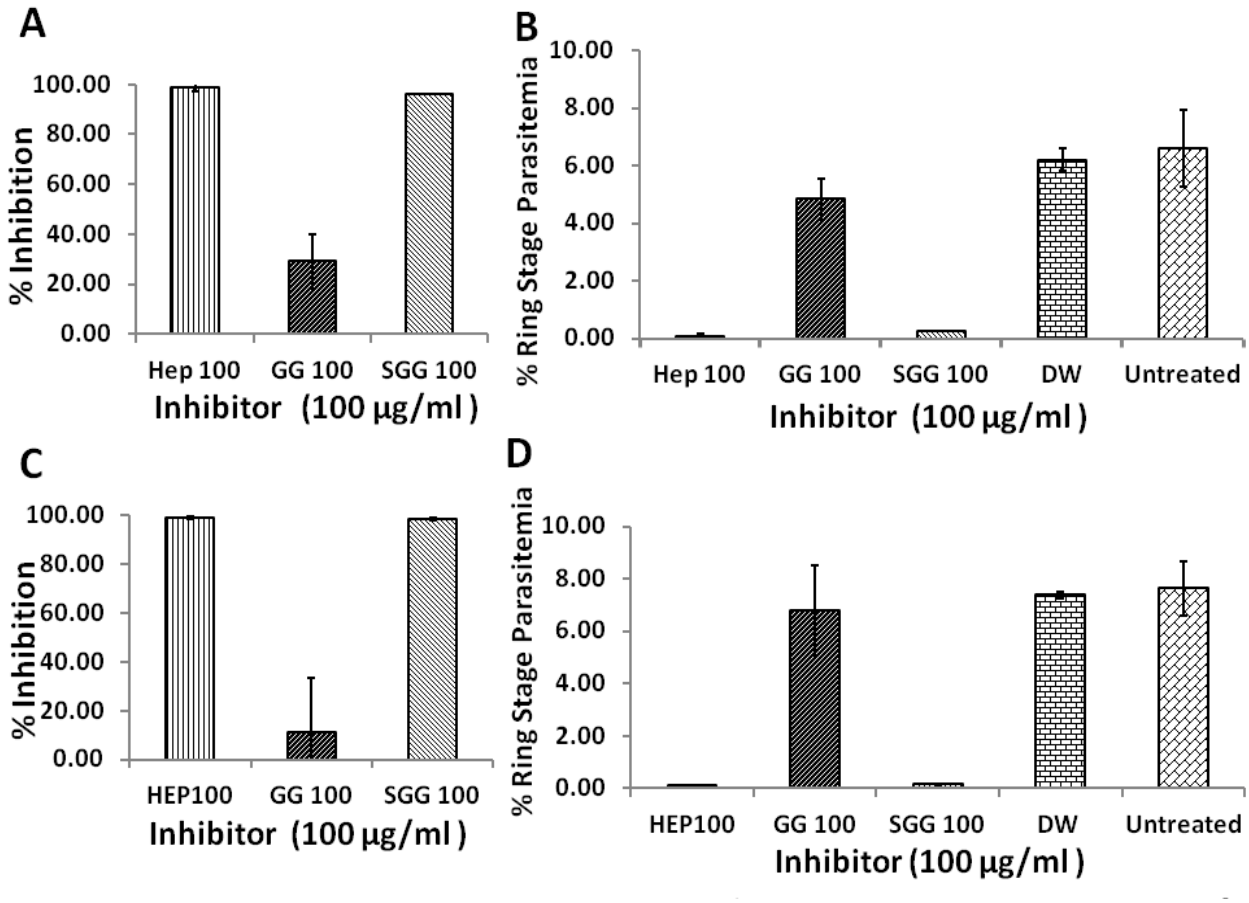
CHAPTER 1

Fig. 3



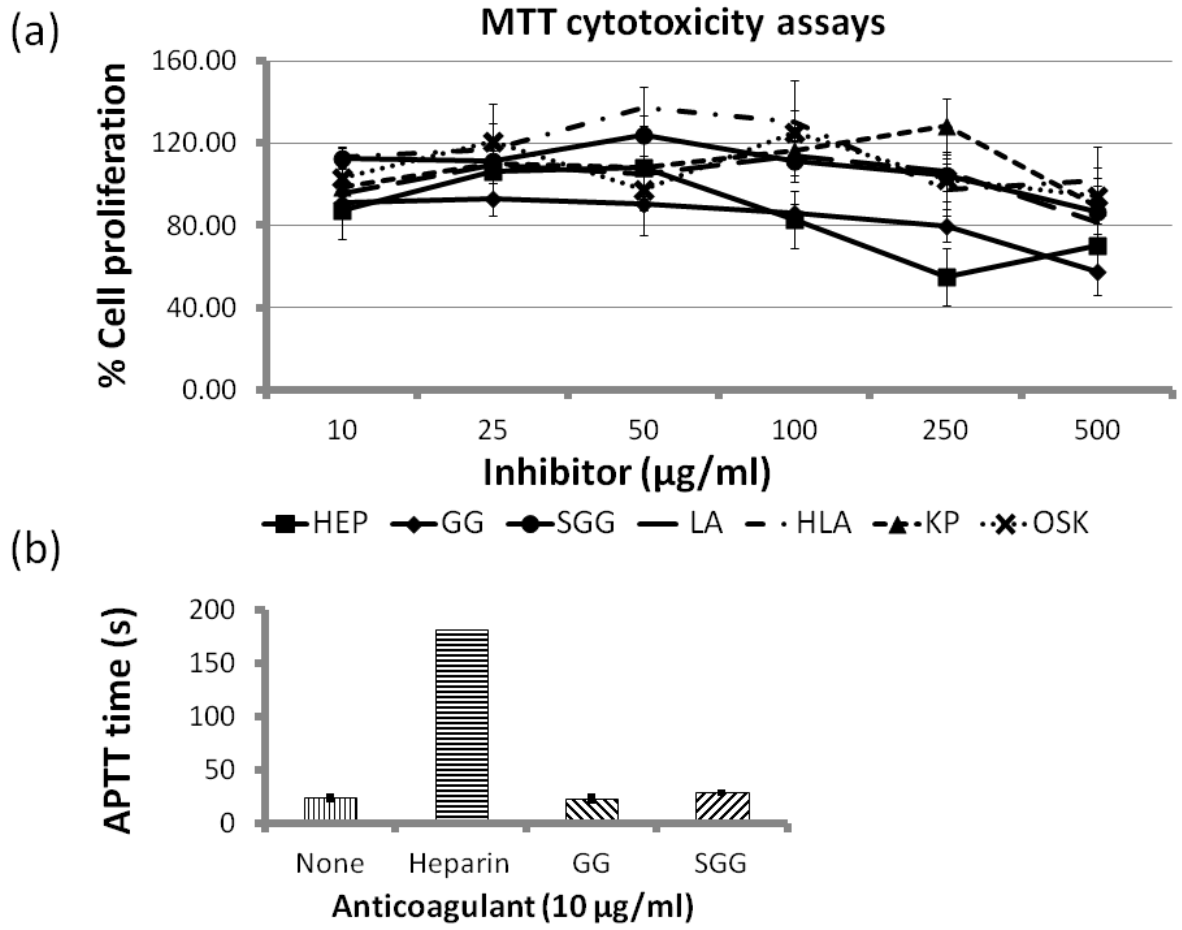
CHAPTER 1

Fig. 4



CHAPTER 1

Fig. 5



CHAPTER 2

Assessment of the *in vivo* inhibition of growth of the rodent malaria parasites, *Plasmodium yoelii* 17XL in BALB/c mice and *Plasmodium berghei* ANKA in C57BL/6 mice using gellan sulfate

Abstract

Our group synthesized gellan sulfate, a novel anticoagulant derivative of the microbial polysaccharide, gellan gum. Gellan sulfate was shown to inhibit the growth and invasion of erythrocytes by the *Plasmodium falciparum* parasites in *in vitro* assays. Also, the synthesized gellan sulfate was found to have low cytotoxicity and anticoagulant activities *in vitro*. Here, the potential of gellan gum as a safer alternative to heparin as adjunct therapy to malaria was further assessed in *in vivo* experiments using the lethal rodent malaria parasites, *Plasmodium yoelii* 17XL in BALB/c mice and *P. berghei* ANKA in C57BL/6 mice following the four day suppressive test. The native gellan gum was tested as well. Gellan sulfate, as well as the native gellan gum, showed low inhibition of the growth rate of both *Plasmodium yoelii* 17XL in BALB/c mice and *P. berghei* ANKA in C57BL/6 mice. However, there was a delay in the mortality of the C57BL/6 mice infected with *P. berghei* ANKA that were treated with both gellan sulfate and the native gellan gum.

1. Introduction

Current antimalarial drugs act on intra-erythrocytic *Plasmodium* parasites [Wilson 2013]. At this stage, the disease is already manifested with signs like fever and malaise, and diagnosis can be done with parasites demonstrated in blood smears.

Quinine, one of the oldest antimalarial agents, was extracted from the bark of the Cinchona trees which are native to the Andes in South America. Quinine and quinine derivatives have been synthesized in the laboratory, making it readily available commercially. Its mode of action involves in binding heme in the *Plasmodium* lysosome, inhibiting heme crystallization. [Sullivan, 2012].

Artemisinins are currently the preferred treatment for severe malaria. Artemisinin-based combination therapies (ACTs) are recommended for uncomplicated malaria. Artemisinin was derived from the *Artemisia* plant leaf extracts in China. Similar to quinine, artemisinin derivatives had been synthesized that makes them readily available commercially [Karunajeewa 2012]. The mechanism of action of artemisinins also involves interfering with the hemoglobin uptake and digestion of the parasite [Klonis 2013].

The development of resistance against antimalarials continues to hinder the efforts to eradicate malaria in the world. Recently, the emergence of resistance against artemisinins on the Thai/Cambodia border was reported [Samarasekera 2009]. It is yet unclear whether these resistant parasites have spread to other areas but such development threatens the progress of the control and elimination of malaria. Hence, the continuing trials to develop preventive vaccines and novel drugs are needed.

Heparin, a highly sulfated polysaccharide, which binds to several proteins of the malaria parasites, inhibits the entry of the parasites into the red blood cells *in vitro* [Wilson 2013]. In addition, heparin can also inhibit cytoadherence and sequestration of infected red blood cells to the vascular endothelium [Xiao 1996] and its low molecular weight derivatives can also prevent rosetting *in vitro* [Skidmore 2008]. However, heparin is not safe for use in clinical malaria because of its anticoagulant properties that can cause intracranial bleeding to the infected individual [WHO 2010]. Other sulfated polysaccharides like dextran sulfate [Xiao 1996], carrageenans [Adams 2005], and fucoidan [Chen 2009] have been found to inhibit the parasite entry into red blood cell *in vitro*. But the effectiveness *in vivo* of these polysaccharides is still inconclusive [James and Alger 1981; Chen 2009; Beuria 1991].

Heparin derivatives are also gaining interest as a safer alternative to heparin that can be used in clinical malaria. One example of which is the depolymerized heparin glycans that lack anticoagulant activity and were shown to release sequestered *P. falciparum* iRBCs in *Macaca fascicularis* [Vogt, et al. 2006]. Blood-stage antimalaria vaccines designed to prevent parasite entry into the red blood cells are still currently under testing. In this light, I wanted to assess the *in vivo* effect of the gellan sulfate, synthesized in our laboratory, which was earlier found to not only effectively inhibit parasite entry and growth *in vitro*, but also to be non-cytotoxic and weakly anticoagulant in *in vitro* tests.

2. Materials and Methods

2.1. Parasites

Frozen stock of *Plasmodium yoelii* 17XL parasites was provided by Prof. Osamu Kaneko (Nagasaki University) and was passaged in BALB/c mice. Frozen stock of *P. berghei* ANKA parasites was provided by Dr. Alaa Terkawi in National Research Center for Protozoan Diseases (NRCPD), Obihiro University of Agriculture and Veterinary Medicine, and was passaged in C57BL/6 mice.

2.2. Animals

Female BALB/c and C57BL/6 mice, five weeks of age were purchased from CLEA Japan (Tokyo, Japan). The animals were maintained in the animal care facility in NRCPD, Obihiro University of Agriculture and Veterinary Medicine under controlled conditions and were given commercial feeds and water ad libitum. The protocol for the animal experiments was approved by the Committee on the Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit No. 25-153, 2013).

2.3. Compounds

Artesunate (SIGMA) was dissolved in 1% sodium bicarbonate in PBS (2 mg/ml) and filter-sterilized. Gellan gum (WAKO) and gellan sulfate were both dissolved in PBS (2 mg/ml) and filter-sterilized.

2.4. Infection and Treatment

Donor mice were infected intraperitoneally with 10^6 parasitized red blood cells from cryopreserved stock. *P. yoelii* 17XL infected blood from the BALB/c donor mice were collected when parasitemia reached 15%. P. *berghei* ANKA infected blood from the C57BL/6 donor mice were collected at 6% parasitemia before presumed onset of cerebral malaria.

Experimental mice were infected intraperitoneally with 10^6 parasitized red blood cells and were sorted into four treatment groups: control untreated, artesunate-treated, gellan gum-treated, and gellan sulfate-treated groups. For ethical reasons, control animals were not given any kind of treatment.

Treatment and monitoring protocol followed the four-day suppressive test. Artesunate was given intraperitoneally at a dose of 20 mg/kg. Gellan gum and gellan sulfate were given intraperitoneally at various doses: for the BALB/c mice, 25 mg/kg, and; for the C57BL/6 mice, 20 mg/kg and 50 mg/kg. The day of infection was designated as day 0, and the first treatment was given 2 hours later. Succeeding treatments were given every 24 hours until day 3 post-infection, for a total of four treatments. Parasitemia monitoring was started at day 4 until day 10 post-infection with Giemsa stained thin blood smears. For parasitemia counting, 10 oil immersion fields were counted with 200-300 cells, except for parasitemias approaching zero, in which cases, 100 fields were examined. Weights and demeanor were monitored to check for signs of toxicity from the treatments. Deaths were promptly recorded.

3. Results

Here the antimalarial effects of the synthesized gellan sulfate were assessed using mouse models infected with the lethal parasites *P. yoelii* 17XL and *P. berghei* ANKA. In all *in vivo* experiments, the parasitemia of the gellan sulfate and the gellan gum treated groups after the four-day suppression of growth were similar and sometimes higher than in the control groups.

In the BALB/c mice infected with *P. yoelii* 17XL (Figure 1A), hyperparasitemia was observed in the control (37.88%), gellan gum (28.82%) and gellan sulfate (26.13%) treated mice in as early as day 4 post-infection. There was also an observed decline in parasitemia from day 6 post-infection. However, this may be attributed to the ensuing anemia rather than the actual effect of the treatments.

BALB/c mice suffering from high parasitemia died at days 5 and 6 p.i (Figure 1B), while some mice persisted until day 25 p.i. All artesunate-treated mice survived beyond day 30 p.i. and eventually recovered from the infection.

Similarly, in C57BL/6 mice infected with *P. berghei* ANKA parasitemias of the control and the gellan gum (20 mg/kg) and gellan sulfate (20 mg/kg) treated groups at day 4 p.i. were almost similar (Figure 2A). Also, the increase in parasitemia for these groups was almost the same. However, all mice (n=3) from the control group died at days 5 and 6 p.i. (Figure 2B), while one mortality from both the gellan gum and gellan sulfate treated groups were noted at day 7 p.i. Artesunate-treated mice eventually succumbed to the infection by day 26-28 p.i. while the last gellan gum and gellan sulfate treated mice died at day 35 and 38 p.i, respectively.

When the doses of gellan gum and gellan sulfate were increased to 50 mg/kg, a similar profile of parasitemia was observed in C57BL/6 mice infected with *P. berghei* ANKA (Figure 3A). In this experiment, mice receiving this dose of both gellan gum and gellan sulfate showed

weight loss and ruffled hair. These may be interpreted as signs of toxicity, and indicate that this dose is not safe for the mice. Majority of the mice persisted beyond day 25 p.i (Figure 3B), but all mice had died of the infection by day 31 p.i.

4. Discussion

In spite of showing good inhibition of growth and invasion of *P. falciparum* 3D7 and Dd2 *in vitro*, gellan sulfate was found to be less effective in inhibiting the growth of the rodent malaria parasites *P. yoelii* 17XL and *P. berghei* ANKA *in vivo.* Several factors can be considered to affect the effectiveness of drugs in the animal body. The animal's immune response to the disease itself is a major determinant of the course of the disease and response to treatment. The method of administration and the delivery system affect the absorption and metabolism of drugs.

Gellan gum is currently being utilized as drug delivery system, and is reportedly safe when given orally and parenterally. When mixed in the diet of Swiss Crl mice for 96-98 weeks, gellan gum showed no effects on the body weight or food consumption of the animals, nor were there any observed neoplastic or non-neoplastic changes associated with feeding gellan gum [Shah 2007]. Gellan gum 1% (w/v) discs showed no significant lesions when implanted in the dorsal part of the BALB/c mice for 21 days [Oliveira 2010]. However, there are no reports on the nutritive or therapeutic benefits of gellan gum.

Gellan sulfate, the sulfated derivative of gellan gum, was originally developed as a novel anticoagulant [Miyamoto 2010]. In this study, when gellan gum and gellan sulfate were given at the dose of 50 mg/kg, some signs of possible toxicity were noted, such as weight loss, stunted growth and ruffled hair. Although some of these changes can also be attributed to the ongoing disease itself, when given at a dose of 20-25 mg/kg, the gellan gum and gellan sulfate treated mice appeared normal until the onset of the clinical disease. Based on these observations, I can say that the dose of 20-25 mg/kg, gellan gum and gellan sulfate are safe to be given intraperitoneally.

The survivals of the infected mice varied from 6 days to more than 30 days for both

Plasmodium yoelii 17XL and *Plasmodium berghei* ANKA infections.

Deaths in the *Plasmodium berghei* ANKA infected groups treated with artesunate may be attributed to the limited time of treatment. Oral artesunate given at 10mg/kg/day for 7 days has been shown to prevent parasitemia but with likelihood of recrudescence of parasitemia and development of cerebral malaria [Gumede *et al*. 2003]. A 14-day treatment course of oral artesunate and at 100mg/kg/day completely prevented development of parasitemia and cerebral malaria [Gumede *et al.* 2003]. For the purposes of comparison, to show the suppression of growth of the parasites, I believe it was shown here that artesunate inhibited growth and proliferation of the parasites during the early stages of the disease under drug pressure. The termination of treatment then allowed the parasites to proliferate and the disease was allowed to run its course.

An interesting observation was that some of the *P. berghei* ANKA infected C57BL/6 mice that were treated with gellan gum and gellan sulfate showed signs of cerebral malaria such as hind limb paralysis and head tilting at around day 7 p.i. Afterwards, these mice apparently recovered from CM and persisted for the next 3 weeks. The exact mechanisms of how gellan gum and gellan sulfate prolonged the survival of the infected mice, however, was not examined in this thesis. It may be hypothesized that the gellan sulfate has an inhibitory effect on the cytoadhesion and sequestration of infected red blood cells that is similar to that of heparin and its derivatives. How this applies to the effect of the native gellan gum, which is not sulfated, still needs further investigation.

Figure legends:

Figure 1. (a) Parasitemia and (b) survival monitoring on BALB/c mice infected with *P. yoelii* 17XL. BALB/c mice were infected intraperitoneally with 10^6 *P. yoelii* 17XL infected red blood cells. Groups of 3 mice were given artesunate (20 mg/kg), gellan gum (25 mg/kg) and gellan sulfate (25 mg/kg) intraperitoneally, following the 4-day suppressive test protocol, with treatment 1 given 2 h post-infection and every 24 h thereafter until day 3 p.i. (a) Parasitemia was measured by Giemsa stained thin blood smears from day 4-10 p.i. (b) Mortality and survival of the infected mice were noted. (dpi – day post-infection), (†) indicate mouse that died.

Figure 2. (a) Parasitemia and (b) survival monitoring on C57BL/6 mice infected with *P. berghei* ANKA, using 20mg/kg gellan gum and gellan sulfate. C57BL/6 mice were infected intraperitoneally with 10^6 *P. berghei* ANKA infected red blood cells. Groups of 3 mice were given artesunate (20 mg/kg), gellan gum (20 mg/kg). Gellan sulfate (20 mg/kg) intraperitoneally, following the 4-day suppressive test protocol, with treatment 1 given 2 h post-infection and every 24 h thereafter until day 3 p.i. (a) Parasitemia was measured by Giemsa stained thin blood smears from day 4-10 p.i. (b) Mortality and survival of the infected mice were noted. (dpi – day post-infection), (†) indicate mouse that died

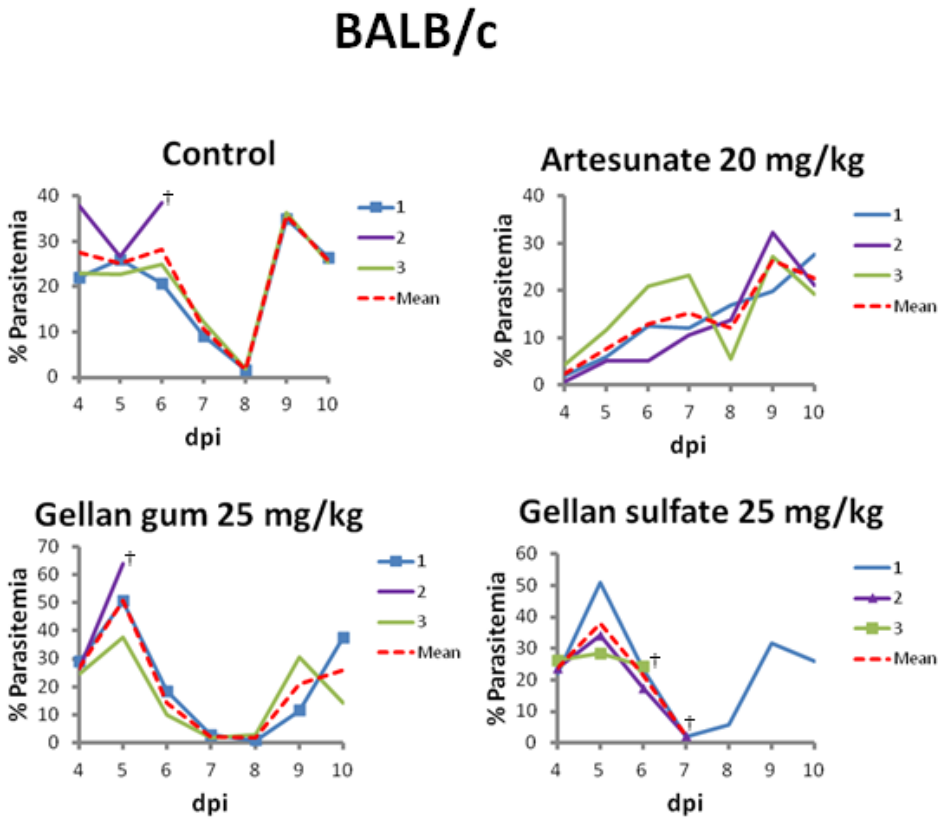
Figure 3. (a) Parasitemia and (b) survival monitoring on C57BL/6 mice infected with *P. berghei* ANKA, using 50 mg/kg gellan gum and gellan sulfate. C57BL/6 mice were infected intraperitoneally with 10^6 *P. berghei* ANKA infected red blood cells. Groups of 3 mice were given artesunate (20 mg/kg), gellan gum (50 mg/kg), and gellan sulfate (50 mg/kg) intraperitoneally, following the 4-day suppressive test protocol, with treatment 1 given 2 h post-

infection and every 24 h thereafter until day 3 p.i. (a) Parasitemia was measured by Giemsa stained thin blood smears from day 4-10 p.i. (b) Mortality and survival of the infected mice were noted. (dpi – day post-infection), (†) indicate mouse that died.

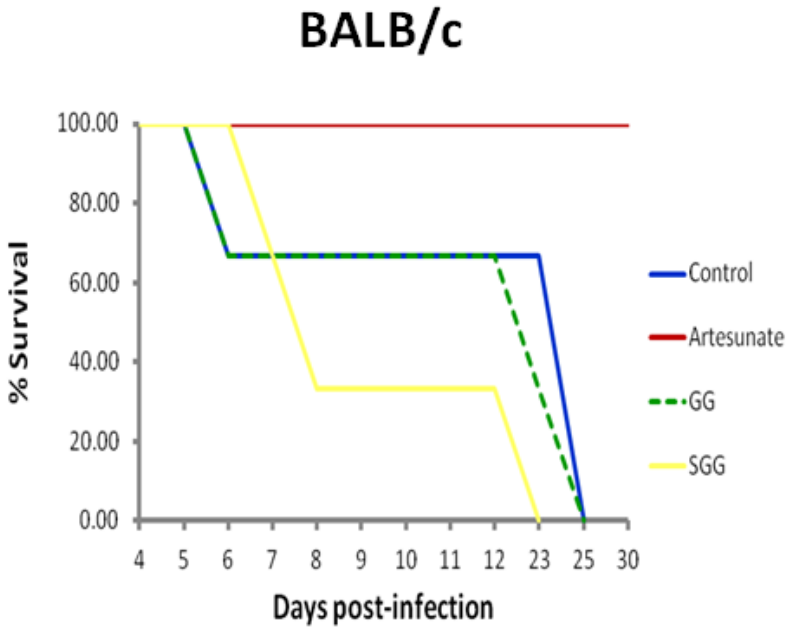
CHAPTER 2

Fig. 1

a

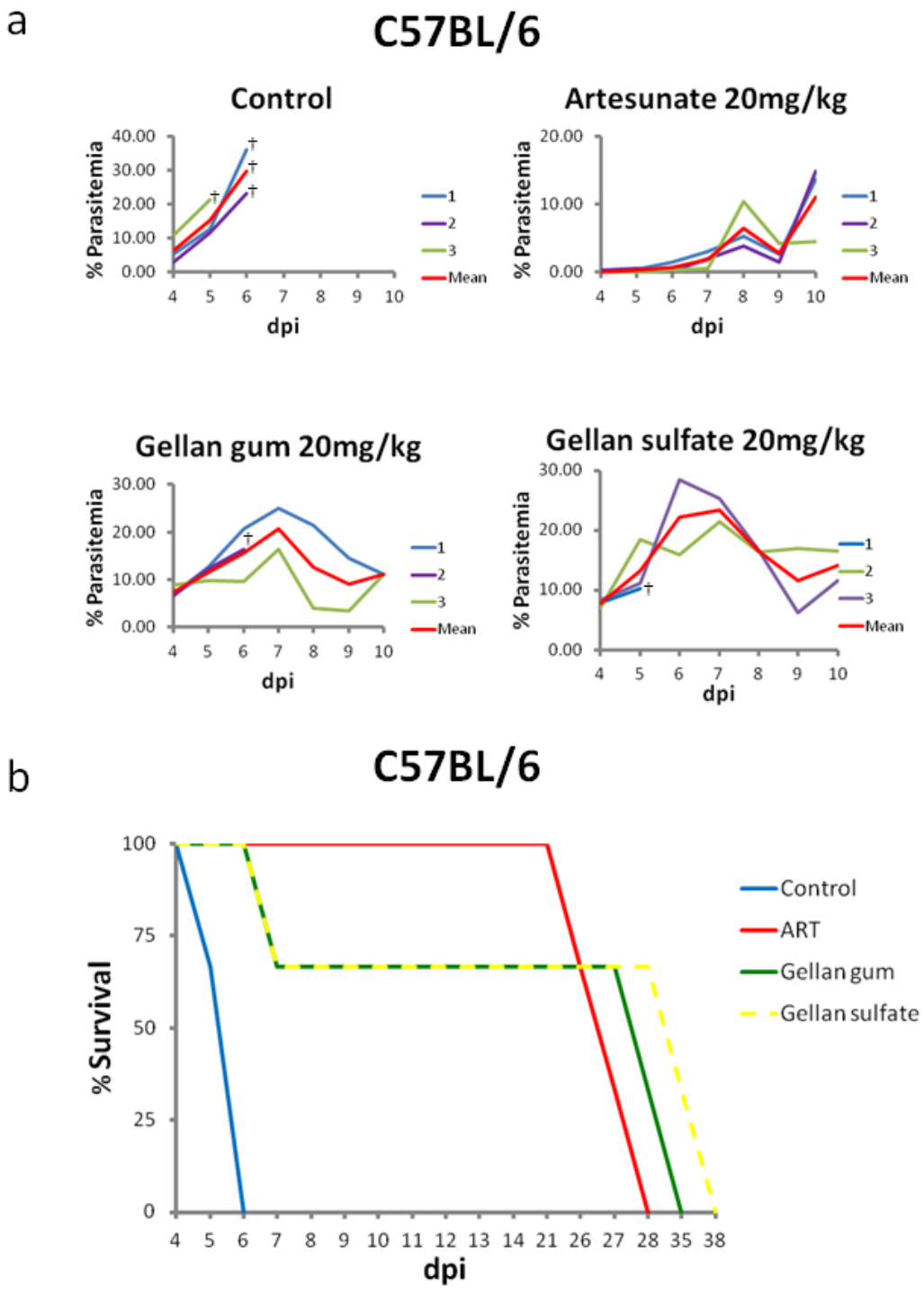


b

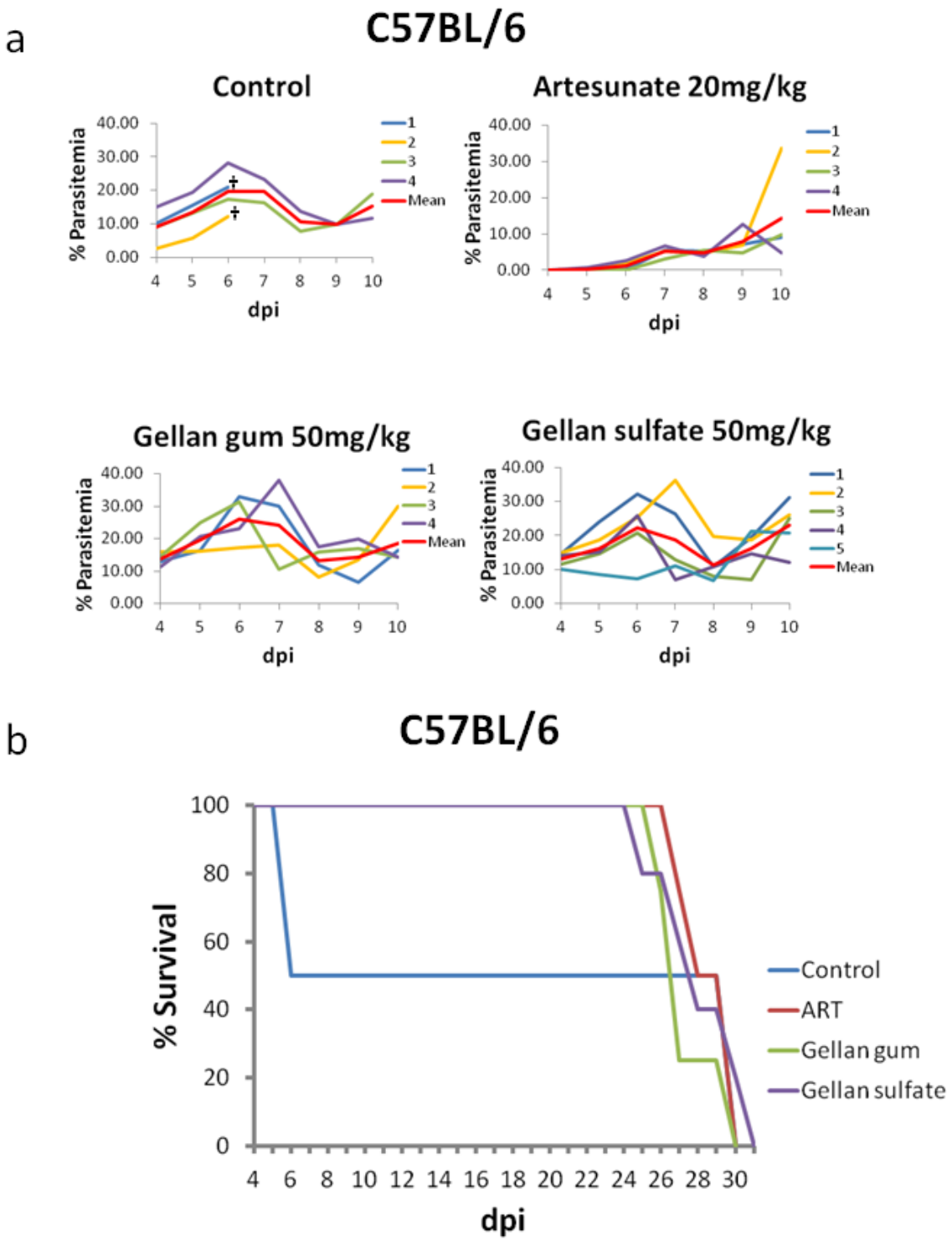


CHAPTER 2

Fig. 2



CHAPTER 2
Fig. 3



CHAPTER 3.

λ- Carrageenan induced experimental cerebral malaria in BALB/c mice infected with *P. berghei* ANKA

Abstract

There is a current interest in novel compounds like carrageenans, which are sulfated polysaccharides derived from seaweeds that have been found to be inhibitory to *P. falciparum in vitro.* It is also important to assess the effects of these substances *in vivo*. In several *in vivo* growth inhibition assays that I did with rodent malaria parasites in mice, I tested several sulfated polysaccharides like fucoidan, dextran sulfate, heparan sulfate and λ- and ι-carrageenan with less than satisfactory results. In addition, in the growth inhibition assay using ι-carrageenan against *P. berghei* ANKA BALB/c mice, one mouse died early in the disease with low parasitemia. I hypothesized that the particular mouse had developed cerebral malaria due to administration of ι-carrageenan. Carrageenans are inflammatory and were even found to alter the permeability of the blood brain barrier in rats. This may add to the development of cerebral malaria. Cerebral malaria (CM) is a severe complication of malaria, and it is of clinical importance with *Plasmodium falciparum* malaria in humans. Rodent models have been beneficial for studying the pathology of CM although it is also highly debated and controversial. Here, I was interested to find out if administration of λ-carrageenan to BALB/c mice, a non-susceptible experimental CM model during malaria infection with *P. berghei* ANKA, can cause the development of cerebral malaria. The results of this study showed that in BALB/c mice infected with *P. berghei* ANKA, lesions in the brain related to cerebral malaria were present. However, in the infected BALB/c mice that were treated with λ-carrageenan, signs of CM such as, head tilt, convulsions and coma were also observed and these mice died earlier than those that received no treatment.

1. Introduction

Carrageenans are linear sulfated polysaccharides derived from seaweeds. The three types of carrageenans are characterized according to their levels of sulfation and are identified by Greek letters. These carrageenan types are: κ-carrageenan, ι-carrageenan, and λ-carrageenan. κ-carrageenan has one sulfate group and forms rigid gels. ι-carrageenan has two sulfate groups and forms soft gels. λ -carrageenan has three sulfate groups and does not gel in solution.

Carrageenans have been found to inhibit the growth and invasion of *P. falciparum* 3D7 and Dd2 *in vitro* [Adams 2005] with λ-carrageenan having the highest inhibitory activity, followed by ι-carrageenan and κ-carrageenan. *In vivo*, calcium carrageenan pre-treatment of A/J mice was shown to decrease the parasitemia and delay death from infection with *P. berghei* NK65 [James 1981].

In several *in vivo* growth inhibition assays on rodent malaria parasites in mice that I did, I tested several sulfated polysaccharides such as fucoidan (Sigma, 100mg/kg BW) [Chen et al. 2009], dextran sulfate (Sigma, 20,000MW; 0.01mg/kg BW) [Beuria and Das, 1991], heparan sulfate (0.10μg/g BW) and λ-carrageenan (Sigma, 20mg/kg BW) [Adams et al. 2005]. In these experiments, I infected BALB/c mice with *P. yoelii* 17XL and gave the sulfated polysaccharides intraperitoneally following the 4-day suppressive test protocol. Parasitemia at day 4 to day 10 post-infection of the mice that were treated with the listed sulfated polysaccharides were slightly but were not significantly lower than that of the untreated mice. The survival of the infected mice was also not improved by treatment of these polysaccharides. I also observed signs of possible toxicity to fucoidan that were not reported in the work by Chen *et al* (2009) which used a similar dose of their own preparation of fucoidan. Immediately after injection of fucoidan

intraperitoneally, the mice were observed to huddle together, appear exhausted, and then later had ruffled hair and weight loss.

In the growth inhibition assay that I did using ι-carrageenan against *P. berghei* ANKA infection in BALB/c mice, one mouse died at day 6 p.i. with 6% parasitemia, while the two others died 2 weeks later. I hypothesized that the particular mouse had developed cerebral malaria due to administration of ι-carrageenan.

Carrageenans are common additives in food and personal hygiene products such as toothpastes and personal lubricants. However, the use of carrageenans as additive in food such as meat and milk has become controversial. Several studies link carrageenans to inflammatory bowel disease and to the development of several allergies and even mammary carcinoma.

Carrageenans have been used to induce inflammation in inflammatory models. Carrageenan-induced inflammation in the paw of mice or rats is a classical model of edema formation and hyperalgesia that is useful in the development of non-steroidal anti-inflammatory drugs. Among the three types of carrageenan, κ-carrageenan was found to be the most inflammatory in the paw edema model while λ-carrageenan had the highest anticoagulant activity *in vitro* [Silva 2010]. In the paw edema model, carrageenans of various concentrations (e.g. 0.1%, 0.5% and 1.0%) dissolved in saline solution are injected into the right hind paw of rats or mice. The animals are given 100 μl of the carrageenan solutions. Similar volume of saline is injected to the left paw as negative control. Paw edema and temperature are checked immediately after injecting the carrageenan and also at certain time points (e.g. 1, 2, 4, 8, 12 and 24h) [Silva 2010]. Histopathology of the paw would reveal edema, tissue injury and a high level of polymorphonuclear neutrophils.

Carrageenan-induced inflammation involves releases of histamine and serotonin followed by release of prostaglandins, protease and lysosomes producing edema [Suralkar 2008]. Another is the carrageenan-induced granuloma pouch model [Suralkar 2008] which is a model of sub acute inflammation. In this method, the animals are fasted overnight, and then the vehicle, the standard and test drugs are administered. A subcutaneous dorsal granuloma pouch is made by injecting 6 ml of air followed by 4 ml of 2% carrageenan in normal saline. Treatment is continued for seven days. The pouch is opened on Day 8. The exudates are collected with a syringe and the volume, the total WBC count and weights of granuloma are determined. Here, formation of exudates with migration of leukocytes and interleukins can be demonstrated.

Since effective malaria vaccines are yet to be developed and distributed to endemic countries and there is a constant threat of the development of resistance of the *Plasmodium* parasites to the current antimalarials and of the *Anopheles* mosquitoes to the common insecticides, it is a continuous effort to discover and develop new antimalarial drugs from novel sources.

Human cerebral malaria (HCM) is the most serious and often fatal complication of severe *P. falciparum* malaria. Patients inflicted with HCM would be in coma showing neurological syndromes including seizures, retinopathy, brainstem alterations and brain swelling. While those who survive HCM develop long-term neurological sequelae, and cognition and behavioral deficits [Renia 2012].

Although the pathogenesis of HCM is still poorly defined, the main cause has been described to involve the sequestration of the infected red blood cells (iRBC) in brain capillaries [Aikawa 1990]. Leukocytes and platelets get sequestered on the endothelium and this leads to

occlusion of brain capillaries that could lead to poor microvascular flow, decreased nutrient supply to the brain and damage to vascular wall that lead to hemorrhages and neuronal alterations [van der Heyde 2006]. Sequestration is a characteristic of P. *falciparum* malaria that involves molecular adhesion between the parasite proteins such as, PfEMP1, the knob protein which is expressed on the surface of the infected erythrocyte and ligands on the endothelial cells, such as CD36, thrombospondin and ICAM-1 [Ponsford 2012].

Experimental cerebral malaria (ECM) can be demonstrated by using the rodent malaria model, *Plasmodium berghei* ANKA in susceptible mouse strains, such as the C57BL/6 mice. However, the use of ECM mouse model is under a lot of controversy because not all aspects of HCM can be reproduced. The most accepted mechanism underlying the development of ECM involves the accumulation of inflammatory cells in the brain, characteristic of encephalitis [Jennings 1996], rather than sequestration of the infected red blood cells.

With the results I gathered from the previous *in vivo* studies, I became interested in the possibility that carrageenan administration to malaria infected mice could induce cerebral malaria. Huber *et al* showed that 72 hour exposure to λ-carrageenan alters the permeability of the blood brain barrier in rats [Huber 2002]. This is a serious side-effect that could compromise the animal.

Here, I was interested to find out if administration of λ-carrageenan to BALB/c mice, a non-susceptible experimental CM model during infection with *P. berghei* ANKA, can cause the development of cerebral malaria. The results of this study showed that in BALB/c mice infected with *P. berghei* ANKA, lesions in the brain related to cerebral malaria were present. However,

the infected BALB/c mice that were treated with λ-carrageenan, showed signs of CM such as, head tilt, convulsions and coma, and these mice died earlier than those that received no treatment.

2. Materials and Methods

2.1. Parasites

Frozen stock of *Plasmodium berghei* ANKA parasites were provided by Dr. Alaa Terkawi in National Research Center for Protozoan Diseases (NRCPD), Obihiro University of Agriculture and Veterinary Medicine and were passaged in BALB/c mice.

2.2. Animals

Female BALB/c mice, five weeks of age were purchased from CLEA Japan (Tokyo, Japan). The animals were maintained in the animal care facility of NRCPD, Obihiro University of Agriculture and Veterinary Medicine under controlled conditions and were given commercial feeds and water ad libitum. The protocol for the experiments was approved by the Committee on the Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit No. 25-153, 2013).

2.3 λ - Carrageenan

λ - carrageenan (SIGMA) was dissolved in PBS (2mg/ml) at 65°C and passed through a 45μm filter (Millipore) for sterilization.

2.4. Infection and Treatment

Donor mice were infected intraperitoneally with 10^6 parasitized red blood cells from cryopreserved stock. When the parasitemia reached around 15%, infected blood was collected by cardiac puncture and mixed with PBS. Two groups of experimental mice were infected intraperitoneally with 10^7 parasitized red blood cells: the first group is the untreated group (PbN group) and the other is the carrageenan-treated group (PbCGN group). Another group of uninfected mice were given λ-carrageenan treatment only (CGN). λ-carrageenan was given intraperitoneally at a dose of 25 mg/kg BW following the four-day suppressive test. In this protocol, infection day is assigned as day 0. The first treatment is given 2 hours after infection, and then subsequent treatments were given every after 24 hours until day 3 post-infection, for a total of 4 treatments.

In independent experiments, half of the infected mice from each group were sacrificed under terminal isoflurane anesthesia at the presumed onset of cerebral malaria for histopathological examination and Evans blue dye assays. While the other half were observed for clinical signs of cerebral malaria until the mice eventually succumb to the disease to monitor the survival rate.

2.5. Parasitemia

Parasitemia levels were monitored from day 4 to day 10 post-infection using Giemsa stained thin blood smears. Ten oil immersion objective fields were examined each with 200-300 red blood cells.

2.6. ECM and survival monitoring

Mice were monitored from day 4 post-infection for general signs such as ruffled fur, and hunching posture, and ECM related signs such as wobbly gait, head tilt, limb paralysis, convulsions, and coma. Other behavioral parameters monitored were reactions to stimuli such as exploration of new environment and touch reflexes [Bopp 2013; Carroll 2010]. Deaths were promptly monitored.

2.7. Histopathology

P. berghei ANKA infected mice were sacrificed under terminal isoflurane anesthesia when either group (PbN or PbCGN) were showing signs of ECM. Brains were examined for gross lesions, photographed and then collected and fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin embedded brains were sectioned at 7 mm. Sections were stained with hematoxylin-eosin (H&E). Uninfected, normal and healthy mice (N) and uninfected, carrageenan-treated mice (CGN) were also sacrificed at the same time for comparison.

2.8. Assessment of vascular leak at the blood-brain barrier by Evans blue dye perfusion.

At day 5 post-infection, 200µl of 1% Evans blue dye was injected into the tail veins of representative mice from each treatment groups. After 1 hr, the mice were sacrificed and the brains were collected and observed for bluish discoloration. The brains were placed in 4%

paraformaldehyde for 48 h to extract the Evans blue dye, and the absorbance was measured at 600nM wavelength.

3. Results

3.1 Effect of λ-carrageenan treatment on parasitemia and course of infection

In this experiment, λ carrageenan showed low inhibition of growth of *P. berghei* ANKA, as shown by hyperparasitemia in the infected mice (Fig. 1A), and did not improve the survival of the infected mice (Fig. 1B). These findings differ from the report by James and Alger (1981) where A/J Swiss mice infected with *P. berghei* NK65 survived up to 28 days when given calcium carrageenan pre-treatment intraperitoneally, and in spite of λ carrageenan showing good inhibition of the growth and invasion of red blood cells of *P. falciparum* in *in vitro* experiments (Adams, 2005).

In the parasitemia, ECM and survival monitoring experiments, all *P. berghei* ANKA infected mice exhibited signs such as ruffled fur, hunching, and decreased reaction to stimuli on day 5 post infection. PbCGN mice had their eyes closed and had prominent third eyelids, and showed limb paralysis, convulsions, head tilt and coma and died soon afterwards. However, on the same day, PbN mice were not observed to exhibit the same neurological signs mentioned. Deaths in the PbN mice group were first seen at day 7 p.i and all PbN mice died by day 13 p.i.

3.2. Gross pathology of brains

PbCGN mice that died on day 5 p.i. from the natural consequences of the disease, as well those sacrificed by overdose of anesthesia, showed hemorrhages on the brains (Figure 2A and 2B). This was not observed in the brains of the PbN mice that died naturally from the disease on day 8 p.i. (Figure 2C). Petechial hemorrhages were observed in the brains of the PbCGN mice showing neurological signs at day 5 p.i. (Figure 2B). The same petechial hemorrhages were also

observed in PbN mice sacrificed on day 5 p.i. (Figure 2D). Here, it was shown that even though the BALB/c mouse is considered resistant, lesions in the brains related to cerebral malaria caused by *P. berghei* ANKA were also observed.

3.3. Histopathology

Brains were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin stain. Histopathological findings are presented in Figure 3. Brain sections from healthy mice showed normal perfusion (Fig. 3A and 3B). Brain sections from carrageenan-treated mice showed no significant lesions (Fig. 3C and 3D). PbN (Fig. 3G and 3H) and PbCGN (Fig. 3F) brains show microhemorrhages and presence of infected red blood cells inside the brain microvessels. Fig. 3E shows brain section from PbN mouse showing apparently normal perfusion.

3.4. Assessment of vascular leak at the blood-brain barrier by Evans blue dye perfusion.

The permeabilty of the blood-brain barrier was assessed by using Evans blue assay. In infected mice that have developed cerebral malaria, the brains would be stained by the blue dye. As shown in Fig. 4, the brains of the PbCGN (Fig. 4C) and PbN (Fig. 4D) mice absorbed the dye indicating increased permeability of the blood-brain barrier compared to that of the brains of normal (Fig. 4A) and carrageenan-treated mice (Fig. 4B). Mean absorbances of the extracted dye from the brains are given in Table 1.

4. Discussion

BALB/c mice was chosen for this study because it is regarded as resistant to the consequences of cerebral malaria caused by *P.berghei* ANKA [Nacer 2012, Renia 2012, Schmidt 2011, Taylor-Robinson 2010]. In this work, λ-carrageenan showed low inhibition of the growth of *P. berghei* ANKA in the BALB/c mice. Survival of the infected mice was not improved with λ carrageenan treatment, and on the contrary, the infected mice treated with λ carrageenan died earlier (day 5) in the infection. Macroscopic evidences of hemorrhages were observed on the brains of the PbCGN mice that died from the natural consequences of the disease, as well as on those sacrificed at the onset of cerebral malaria. Histopathologic examination of the brains of PbCGN mice showed microhemorrhages and sequestration of iRBCs and leukocytes in the brain microvessels.

It was interesting to also observe hemorrhages in both gross and histopathologic examinations of the brains from PbN mice. Neill and Hunt [1992] previously reported that infection with *P.berghei* ANKA led to development of ECM in the BALB/c mice and mortality occurred at 6-8 days post-infection. However, consistent with previous reports about resistance to ECM caused by *P.berghei* ANKA in the BALB/c mice, in some PbN mice normal findings were demonstrated on the brains on both gross and histopathologic examinations.

Leakage of Evans blue dye indicates increased blood brain barrier permeability in both PbCGN and PbN mice. These results suggest that *P. berghei* ANKA infection in itself causes alterations of the blood brain barrier.

On histopathology, there were no significant lesions observed on the brains from the CGN mice, although dark staining bodies, possibly inflammatory cells, or artifacts were observed. In the Evans blue dye assays, there was no bluish staining of the brain in spite of the initial report by Huber *et al* (2002) that CGN administration increases the permeability of the blood brain

barrier. The Evans blue dye injection was done at day 5 p.i., which was 48 hr after the last intraperitoneal injection of λ carrageenan. Whether this time was enough for the blood brain barrier to recover from the alterations caused by carrageenan administration is not clear at this point.

These results show that cerebral malaria caused by *P.berghei* ANKA can develop in the BALB/c mouse and λ-carrageenan treatment can accelerate the development of cerebral malaria and cause acute death in the infected BALB/c mice.

FIGURE LEGENDS

Figure 1. Parasitemia and survival of *P. berghei* ANKA-infected BALB/c mice. (a) Hyperparasitemia observed in both groups of *P. berghei* ANKA-infected mice not treated (PbN) and treated with λ-carrageenan (PbCGN). λ-Carrageenan treatment (25 mg/kg) did not inhibit the growth of *P. berghei* ANKA *in vivo* following the four-day suppressive test. Mouse numbers marked with asterisk (*) indicate the specific mice sacrificed for histopathological examination on day 5 p.i. **(b)** Survival profile of *P. berghei* ANKA-infected BALB/c mice: λ-carrageenan treated (PbCGN); not treated infected mice (PbN). (†) indicate mouse that died from natural consequences of the disease.

Figure 2. Gross lesions on the brains of different treatment groups. (a) Pb-infected λ-carrageenan-treated (PbCGN) mice that died naturally from the infection. Left panel: Unopened braincase showing intracranial bleeding. Right panel: Dissected brains with hemorrhages. **(b)** PbCGN mice that were sacrificed at the onset of cerebral malaria (day 5 post-infection). Left: Unopened brain case showing intracranial bleeding. Center: Brain from PbCGN mouse with petechial hemorrhages. Right: Brain from PbCGN mouse without hemorrhages. **(c)** Pb-infected untreated (PbN) mouse that died naturally on day 8 p.i., brain case opened showing no hemorrhages. **(d)** Brains of PbN mice that were sacrificed at the presumed onset of cerebral malaria (day 5 post-infection). Left: Brain from PbN mouse without hemorrhages. Right: Brain from PbN mouse with petechial hemorrhages. **(e)** Brains from λ–carrageenan treated (CGN) mice showing no significant lesions. **(f)** Brains from uninfected, untreated, healthy (N) mice showing no significant lesions.

Fig. 3. Histopahtology of brains from different treatment groups. (a, b) Uninfected, untreated, healthy (N) mice showed normal brain perfusion. The pictures were taken at 100x and 400x. **(c, d)** λ–carrageenan treated (CGN) mice showing no significant lesions. The pictures were taken at 100x and 400x. **(e)** Brain section of Pb-infected, untreated (PbN) mouse sacrificed at the presumed onset of cerebral malaria (day 5 post-infection), with apparently normal findings and **(f)** from another mouse showing hemorrhagic lesions. The pictures were taken at 100x. **(g, h)** Pb-infected, CGN-treated mouse (PbCGN) that was sacrificed at the onset of cerebral malaria (day 5 post-infection), showing hemorrhage and hemozoin deposits in parasitized red blood cells inside the brain capillaries (shown with arrows). The pictures were taken at 400x.

Fig. 4. Assessment of vascular leak at the blood-brain barrier by Evans blue dye perfusion. Two hundred microliters of 1% Evans blue dye was injected into the tail veins of each mice at Day 5 post-infection. After 1 hr, the mice were sacrificed using terminal ether anesthesia and the brains were collected. Representative brains are shown as follows: **(a)** Normal healthy mouse (N), **(b)** Carrageenan-treated mice (CGN), **(c)** *P. berghei* ANKA infected, carrageenan treated mice (PbCGN), **(d)** and **(e)** *P. berghei* ANKA infected, untreated mice (PbN).

CHAPTER 3

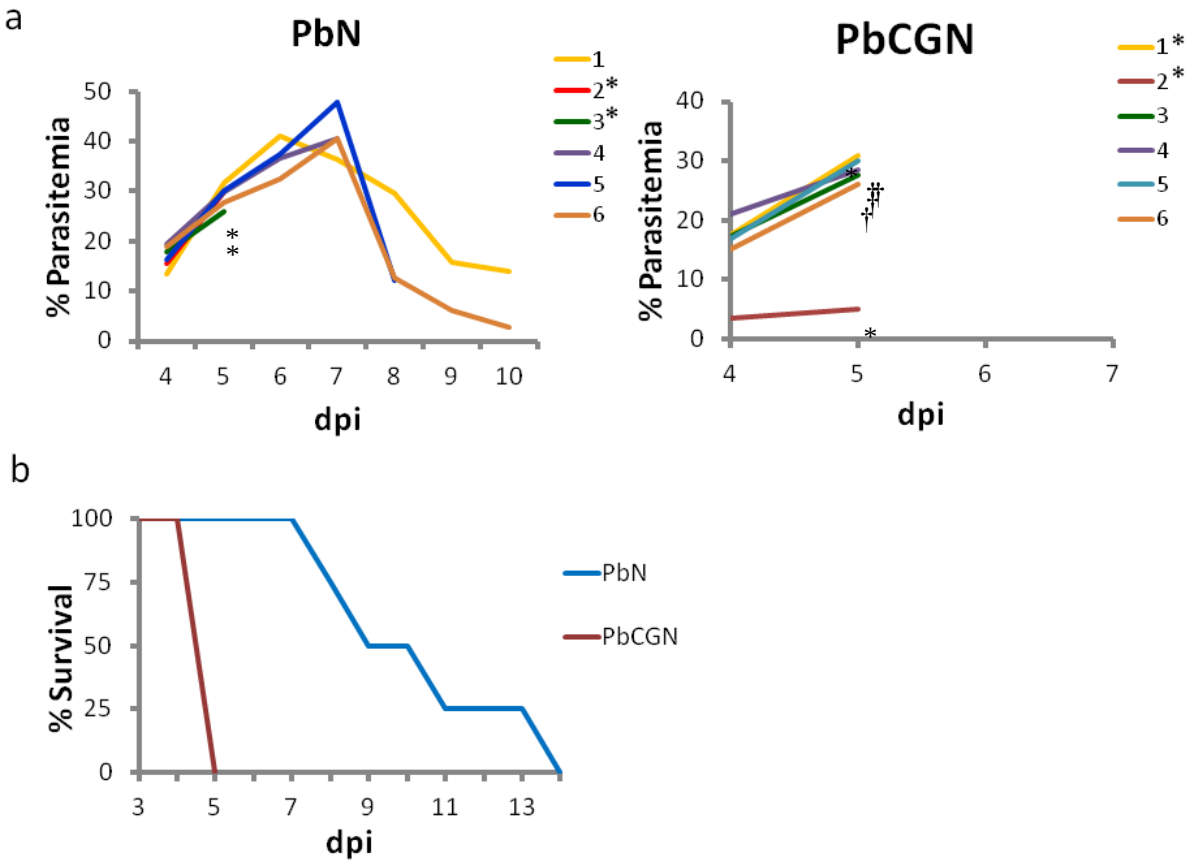
Table 1. Evans blue dye extraction assay

Table 1. Evans ble dye extraction assay		
Mouse	**Mean Abs $_{600NM}$**	**SD**
Normal	0.039	0.001
CGN1	0.060	0.001
CGN2	0.059	0.002
PbN1	0.091	0.003
PbN2	0.074	0.003
PbCGN1	0.077	0.005
PbCGN2	0.067	0.001

Mean absorbances at 600nM wavelength and standard deviations of extracted dye after placing the brains in 4% paraformaldehyde for 48 h showing high dye absorption of brains from the PbN and PbCGN mice.

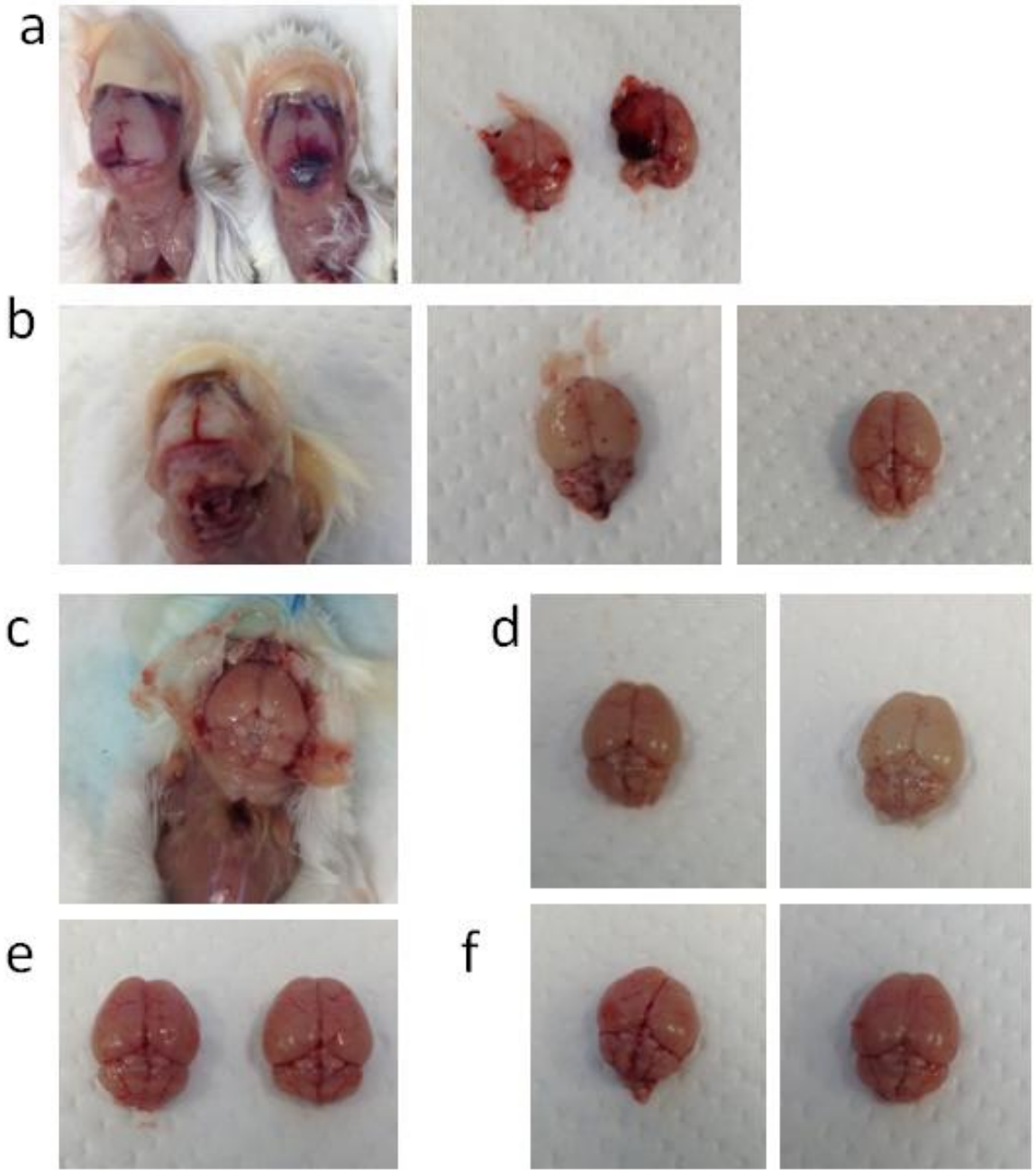
CHAPTER 3

Fig. 1



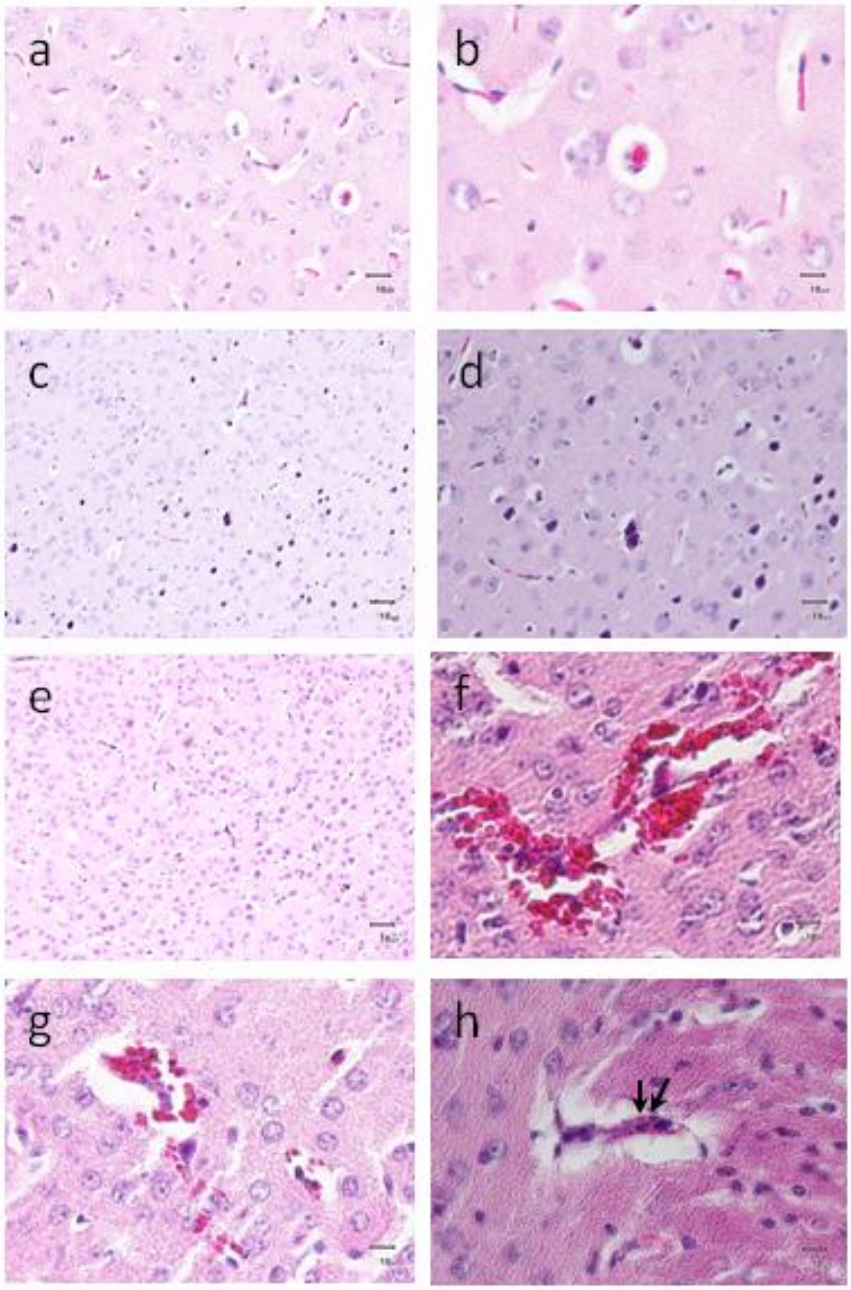
CHAPTER 3

Fig. 2



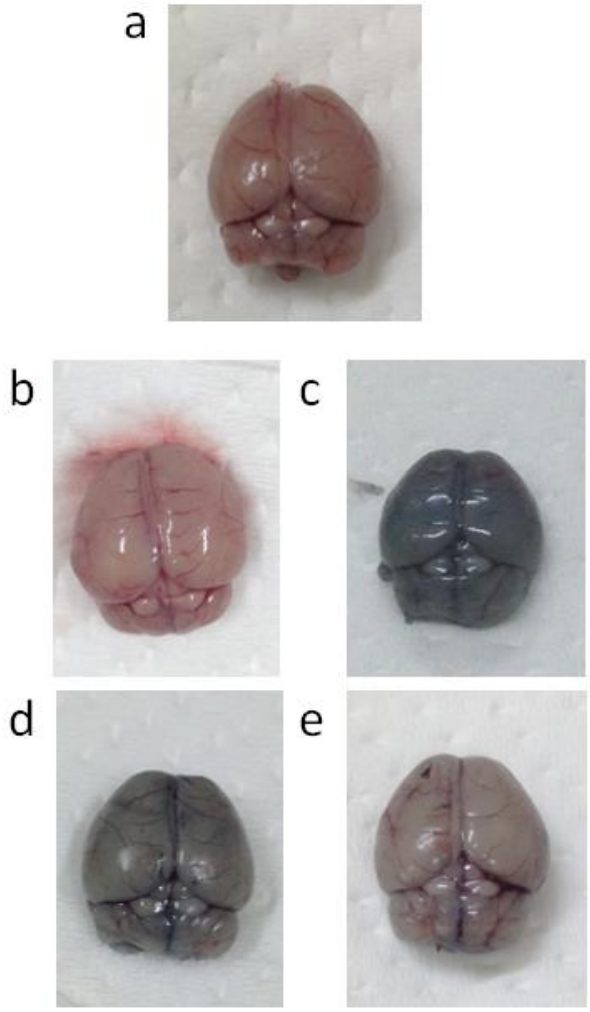
CHAPTER 3

Fig. 3



CHAPTER 3

Fig. 4



GENERAL CONCLUSION

In chapter 1, I described the detail of the synthesis of modified polysaccharides, namely: oversulfated κ- and hydrolyzed λ-carrageenans, and gellan sulfate, the sulfated derivative of the microbial polysaccharide gellan gum. Nuclear magnetic resonance spectroscopy and elemental analysis showed successful sulfation of κ-carrageenan and gellan gum. These derivatives were then evaluated for their activities against the human malaria parasites, *P. falciparum, in vitro.* Here, I used two *P. falciparum* lines namely, 3D7, the chloroquine sensitive line and Dd2, the chloroquine resistant line. While most of the antimalarials currently in use act on the parasite after entry into the erythrocyte, the highly sulfated glycosaminoglycan, heparin inhibits parasite entry. However, heparin use in clinical malaria causes bleeding as side effect. As an alternative, compounds with structures similar to heparin, such as heparan sulfate and its low molecular weight derivatives, as well as other sulfated polysaccharides, such as fucoidan, dextran sulfate, and carrageenans and their derivatives, can be exploited in finding compounds that can inhibit parasite entry into the host erythrocyte.

Gellan sulfate was shown to inhibit the growth and invasion of *P. falciparum in vitro*. The oversulfated κ- and hydrolyzed λ-carrageenans, however, showed poor inhibition of *P. falciparum in vitro*. These derivatives were also found to be non-cytotoxic based on *in vitro* MTT cytotoxicity assays on 293T cells. With further interest in gellan sulfate, I performed APTT anticoagulant assays *in vitro* and found that it has low anticoagulant activity.

In chapter 2, the synthesized gellan sulfate was assessed for its *in vivo* ability to suppress the growth of *P. yoelii* 17XL and *P. berghei* ANKA, in the BALB/c and C57BL/6 mice, respectively. Following the 4-day suppressive test, the mice were injected with parasites intraperitoneally at day 0 and were given treatments of artesunate, gellan gum and gellan sulfate

at various doses by the same route from day 0 to day 3 post-infection. Parasitemias from day 4 to day 10 post-infection showed no difference between the control and gellan sulfate treated groups. This suggests that gellan sulfate, which showed good inhibition of growth and invasion of *P. falciparum* 3D7 and Dd2 *in vitro*, had little effect on the growth of *P. yoelii* 17XL and *P. berghei* ANKA *in vivo*. It was also observed that some infected mice that were treated with both the native gellan gum and gellan sulfate survived until 30 days post-infection, showing a delay in the death of the infected mice. It can be assumed that gellan sulfate may have inhibitory effect similar to heparin, on the sequestration and cytoadhesion of infected red blood cells which leads to the more serious consequences of malaria, such as cerebral malaria and placental malaria. However, it was unclear why the same effect on survival was observed in the group that was given native gellan gum because of the limited studies that focus on the physiological effects of gellan gum other than it being safe for use as a drug vehicle. It was also shown here that the native gellan gum and gellan sulfate are safe to be given intraperitoneally at dose of up to 25mg/kg bodyweight but may be toxic at dose of 50mg/kg BW.

In chapter 3, the effect of λ carrageenan on the development of experimental cerebral malaria (ECM) in mice was investigated. Here, I had intended to show that since λ-carrageenan injection in rats has been shown to alter the permeability of the blood brain barrier, experimental cerebral malaria may develop in the BALB/c mouse, which is considered resistant to ECM caused by *P. berghei* ANKA. This is in contrast to the C57BL/6 mouse, which is considered a susceptible strain, and in which strain, signs of ECM can be clearly observed and mortality due to ECM occurs at day 6 or 7 post infection.

Following the 4-day suppressive test protocol, λ-carrageenan was given intraperitoneally to BALB/c mice infected with *P. berghei* ANKA. Carrageenans had been reported to inhibit *P.*

falciparum 3D7 and Dd2 *in vitro.* But in this study, parasitemias of the infected mice rose at the same rate for the control and λ-carrageenan-treated groups. This shows that λ-carrageenan had little effect on the growth of *P. berghei* ANKA *in vivo*.

The results also showed that the BALB/c mice infected with *P.berghei* ANKA, both the λ-carrageenan treated (PbCGN) and untreated groups (PbN), showed leakage of the blood brain barrier and histopathologic lesions, such as focal and microhemorrhages in the brain, that are used to describe ECM. Clinical signs of cerebral malaria like convulsions and limb paralysis were not observed in mice untreated with λ-carrageenan, but signs, which overlap with those of severe malaria, were observed for both treatment groups. These signs included shivering and huddling, weakness, and reluctance to move. Deaths occurred earlier in the λ-carrageenan-treated group. The PbCGN mice died within 5 days post-infection after having seizures and falling into moribund state. These results indicate that λ-carrageenan administration exacerbate malaria by inducing the development of cerebral malaria leading to acute death of the animal.

It became interesting for me to study the effects of λ-carrageenan on the possible development of cerebral malaria in the BALB/c mice infected with *P. berghei* ANKA because prior to making the modified polysaccharides, I performed *in vivo* growth inhibition assays on *P. yoelii* 17XL using different sulfated polysaccharides, namely: fucoidan (Sigma, 100mg/kg BW), dextran sulfate (Sigma, 20,000MW; 0.01mg/kg BW), heparan sulfate (0.10μg/g BW) and λ-carrageenan (Sigma, 20mg/kg BW) in BALB/c mice. In the above-mentioned experiments, the parasitemia of the infected mice that were treated with the aforementioned sulfated polysaccharides were slightly but not significantly lower than that of the untreated mice. Survival of the infected mice was not improved by treatment of these polysaccharides as well. In addition, I observed that immediately after injection of fucoidan (100mg/kg) intraperitoneally,

the mice huddled together and then had ruffled hair and weight loss. These signs, which can be interpreted as signs of toxicity to the drug, were not reported in the work by Chen et al (2009) which used a similar dose of their own preparation of fucoidan.

In another experiment, I used ι-carrageenan against *P. berghei* ANKA in BALB/c mice. One in three mice died at day 6 post-infection with parasitemia of 6% , while the two others died 2 weeks later. This was in spite of ι-carrageenan being considered to be the safest to administer intraperitoneally among the 3 types of carrageenan [Thomson and Horne, 1976]. The report that λ-carrageenan injection in rats alters the permeability of the blood brain barrier [Huber, 2002] led me to hypothesize that the cause of death for that specific mouse was possibly due to the development of cerebral malaria.

This also prompted my interest to study as to whether the modification of polysaccharides, such as sulfation of κ-carrageenan could lead to antimalarials that inhibit entry of the *Plasmodium* merozoites *in vitro*, similar to heparin, but are safer for use in clinical malaria.

This work shows that naturally-derived or synthetic novel sulfated polysaccharides are promising sources of new antimalarials which have modes of action similar to heparin, inhibiting the invasion of red blood cells by the *Plasmodium* parasites, but with less anticoagulant activity. If the invasion process is halted, there is a chance that the immune system targets the free *Plasmodium* merozoites, eventually leading to clearance of the parasites and development of immunity against malaria infection. However, at present, there is yet to be such glycosaminoglycan that is safe for use in clinical malaria. As shown here, gellan sulfate and carrageenans which were effective against malaria parasites *in vitro* were less effective against

malaria parasites *in vivo*. It was also shown here that carrageenans can also cause severe complications during malaria infection.

The recent work by Mathias *et al* [Mathias *et al* 2013] showing the potential of a synthetic glycosaminoglycan as a transmission blocking drug, opens another avenue for the application of these sulfated polysaccharides on the control of malaria that is worth exploring in the future.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all the people who helped me in my studies.

I am entirely grateful to my supervisors and professors for their guidance and patience throughout this undertaking. This work benefitted much from their comments and suggestions:

Dr. Kentaro Kato (Obihiro University of Agriculture and Veterinary Medicine, The University of Tokyo)

Dr. Taisuke Horimoto (The University of Tokyo)

Dr. Hiroomi Akashi (The University of Tokyo)

Dr. Yasuyuki Goto (The University of Tokyo)

Dr. Yasuhiro Takashima (Gifu University)

Dr. Kyousuke Kobayashi (The University of Tokyo)

Dr. Tadahisa Iwata (The University of Tokyo)

Dr. Yukiko Enomoto-Rogers (The University of Tokyo)

Dr. Noriyuki Horiuchi (Obihiro University of Agriculture and Veterinary Medicine)

Dr. Mizuki Tomihari and Mr. Kazuro Miyahara (Obihiro University of Agriculture and Veterinary Medicine)

Dr. Maria Shirley Herbas (Obihiro University of Agriculture and Veterinary Medicine)

I am also thankful for all the help provided by Ms. Kyoko Kano and Ms. Akiko Okuyama.

I wish to express my appreciation to all the members of the Department of Veterinary Microbiology, Graduate School of Agriculture and Life Sciences, The University of Tokyo and also of Research Unit for Global Infection Control, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine. With special mention to Dr. Akiko Ishiwa and my classmate, Mr. Tatsuki Sugi for all their help and suggestions.

A warm thanks to my family and friends here in Japan and back home in the Philippines, especially my sister for her encouragement and support for the completion of this work.

And I would like to thank God, in whom all things are possible, for all His blessings. All glory and honor belong to Him.

REFERENCES

Abraham, E. G., Jacobs-Lorena, M. (2004). Mosquito midgut barriers to malaria parasite development. *Insect Biochemistry and Molecular Biology,* **34** (7), 667–671.

Achur R. N., Valiyaveettil M., Alkhalil A., Ockenhouse C. F., Gowda D. C. (2000). Characterization of proteoglycans of human placenta and identification of unique chondroitin sulfate proteoglycans of the intervillous spaces that mediate the adherence of *Plasmodium falciparum*-infected erythrocytes to the placenta. *The Journal of Biological Chemistry*, **275** (51), 40344-40356.

Adams Y., Smith S. L., Schwartz-Albiez R., Andrews K.T. (2005). Carrageenans inhibit the *in vitro* growth of *Plasmodium falciparum* and cytoadhesion to CD36. *Parasitology Research,* **97**, 290-294.

Barnes, K. I. (2012). Antimalarial drugs and the control and elimination of malaria. In: *Treatment and Prevention of Malaria*. Staines, H.M. and Krishna, S. (eds.). (pp. 1-13). AG: Springer Basel.

Bates, A. H., Mu, J., Jiang, H., Fairhurst, R. M., Su, X. Z. (2010). Use of magnetically purified *Plasmodium falciparum* parasites improves the accuracy of erythrocyte invasion assays. *Experimental Parasitology*, **126**, 278-280.

Beuria M. K., Das M. K. (1991). Dextran sulfate induced suppression of *Plasmodium berghei* parasitaemia. *Indian Journal of Experimental Biology*, **29** (3), 284-285.

Bo S., Muschin T., Kanamoto T., Nakashima H., Yoshida T. (2013). Sulfation and biological activities of konjac glucomannan. *Carbohydrate Polymers*, **94**, 899-903.

Bopp, S. E. R., Rodrigo, E., Gonzalez-Paez, G. E., Frazer M., Barnes S. W., Valim C., Watson J., Walker J. R., Schemdt C., Winzeler E. A. (2013). Identification of the *Plasmodium beghei* resistance locus 9 linked to survival on chromosome 9. *Malaria Journa,* **12**, 316.

Boyle M. J., Richards J. S., Gilson P. R., Chai W., Beeson J. G. (2010). Interactions with heparin-like molecules during erythrocyte invasion by *Plasmodium falciparum* merozoites. *Blood,* **115**, 4559-4568.

Carrara V. I., Lwin K. M., Phyo A.P., Ashley E., Wiladphaingern J., Sriprawat K., Rijken M., Boel M., McGready R., Proux S., Chu C., Singhasivanon P., White N., Nosten F. (2013). Malaria burden and artemisinin resistance in the mobile and migrant population on the Thai–Myanmar border, 1999–2011: an observational study. *PLoS Medicine,* **10** (3): e1001398. doi:10.1371/journal.pmed.1001398.

Carroll, R.W., Wainwright M.S., Kim, K.Y., Kidambi T., Gomez, N. D., Taylor T., Haldar K. (2010). A rapid murine coma and behavior scale for quantitative assessment of murine cerebral malaria. *PLOS One,* **5**, e13124.

Chen J. H., Lim J. D., Sohn E. H., Cho Y. S., Han E. T. (2009). Growth-inhibitory effect of a fucoidan from brown seaweed *Undaria pinnatifida* on *Plasmodium* parasites. *Parasitology Research,* **104**, 245-250.

Clark D. L., Su S., Davidson E. A. (1997). Saccharide anions as inhibitors of the malaria parasite. *Glycoconjugate Journal*, **14**,473-79.

Cowman A., Berry D., Baum J. (2012). The cellular and molecular basis for malaria parasite invasion of the human red blood cell. *The Journal of Cell Biology*, **198**, 961-971.

de Araujo, C. A., Noseda, M. D., Cipriani, T.R., Goncalves, A.G., Duarte, M.E.R. and Ducatti, D.R.B. (2013). Selective sulfation of carrageenans and the influence of sulfate regiochemistry on anticoagulant properties. *Carbohydrate Polymers*, **91** (2), 483-491.

Edmondson J. M., Armstrong L. S., Martinez A. O. (1988). A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. *J. Tissue Culture Methods*, **11**, 15-17.

Gamache D. A., Povlishock J. T., Ellis E. F. (1986). Carrageenan-induced brain inflammation. Characterization of the model. *Journal of Neurosurgery*, **65** (5), 679-685.

Garman R. H. (2011). Histology of the central nervous system. *Toxicologic Pathology*, **39**, 22-35,

Gaur D., Chitnis C. E. (2011). Molecular interactions and signaling mechanisms during erythrocyte invasion by malaria parasites. *Current Opinion in Microbiology*, **14**, 422-428.

Goncalves V. M. F., Reis A., Domingues M. R. M., Lopes-da-Silva J. A., Fialho A. M., Moreira L. M., Sa-Correira I., Coimbra M. A. (2009). Structural analysis of gellans produced by *Sphingomonas elodea* strains by electrospray tandem mass spectrometry. *Carbohydrate Polymers*, 77, 10-19.

Grellier P., Deregnaucourt C., Florent I. (2012). Advances in Antimalarial Drug Evaluation and New Targets for Antimalarials. In: *Malaria Parasites*. Okwa O. (Ed.), ISBN: 978-953-51-0326-4. http://www.intechopen.com/books/malaria-parasites/advances-in-antimalarial-drug-evaluation-and-new-targets-for-antimalarial-chemotherapy.

Gumede B., Folb *., Ryffel B. (2003) Oral artesunate prevents *Plasmodium berghei* Anka infection in mice. *Parasitology International,* **52**, 53-59.

Harvey K. L., Gilson P. R., Crabb B. S. (2012). A model for the progression of receptor-ligand interactions during erythrocyte invasion by *Plasmodium falciparum*. *International Journal of Parasitology*, **42**, 567-573.

Huber J. D., Hau V. S., Borg L., Campos C. R., Egleton R. D., Davi, T. P. (2002). Blood-brain barrier tight junctions are altered during a 72-h exposure to λ-carrageenan-induced inflammatory pain. *Am J Physiol Heart Circ Physiol*, **283**, H1531 - H1537.

Igweh J. C. (2012). Biology of Malaria Parasites. In: *Malaria Parasites*. Okwa O. (Ed.), ISBN: 978-953-51-0326-4, InTech, DOI: 10.5772/34260. http://www.intechopen.com/books/malaria-parasites/biology-of-malaria-parasites

Itoh M., Takasaki I., Andoh T., Nojima H., Tominaga M., Kuraishi Y. (2001). Induction by carrageenan inflammation of prepronociceptin mRNA in VR1-immunoreactive neurons in rat dorsal root ganglia. *Neuroscience Research*, **40** (1), 227-233.

James M. A., Alger N. E. (1981). *Plasmodium berghei*: effect of carrageenan on the course of infection in the A/J mouse. *International Journal for Parasitology*, **11**, 217-220.

Jay, A. J., Colquhoun, I. J., Ridout, M. J., Brownsey, G. J., Morris, V. J., Fialho, A. M., Leitao, J. H., Sa-Correia, I. (1998). Analysis of structure and function of gellans with different substitution patterns. *Carbohydrate Polymers* **35** (3-4), 179-188.

Jennings V. M., Actor J. K., Lal A. A., Hunter R. L. (1997). Cytokine profile suggesting that murine cerebral malaria is an encephalitis. *Infection and Immunity*, **65** (11), 4883-4887.

Karunajeewa, H. (2012). Artemisinins: artemisinin, dihydroartemisinin, artemether and artesunate. In: *Treatment and Prevention of Malaria* (pp. 157-183). AG: Springer Basel.

Kawahara S., Yoshikawa A., Hiraoki T., Tsutsumi A. (1996). Interactions of paramagnetic metal ions with gellan gum studied by ESR and NMR methods. *Carbohydrate Polymers,* **30**, 129-133.

Kherani Z. S., Auer R. N. (2008). Pharmacologic analysis of the mechanism of dark neuron production in cerebral cortex. *Acta Neuropathologica*, **116** (4), 447-52.

Klonis, N., Creek D. J., Tilley L. (2013). Iron and heme metabolism in *Plasmodium falciparum* and the mechanism of action of artemisinins. *Current Opinion in Microbiology*, **16** (6), 722-727.

Kobayashi K., Kato K., Sugi T, Takemae H., Pandey K., Gong H., Tohya Y., Akashi H. (2010). *Plasmodium falciparum* BAEBL binds to heparan sulfate proteoglycans on the human erythrocyte surface. *The Journal of Biological Chemistry*, **285**, 1716-1725.

Kulane A., Ekre H. P., Perlmann P., Rombo L., Wahlgren M., Wahlin B. (1992). Effect of different fractions of heparin on *Plasmodium falciparum* merozoite invasion of red blood cells *in vitro*. *American Journal of Tropical Medicine and Hygiene,* **46,** 589-594.

Kweka E. J., Mazigo H. D., Munga S., Magesa S. M., Mboera L. E. G. (2013). Challenges to malaria control and success stories in Africa. *Global Health Perspectives*, **1**, 71-80.

Lobo C. A., Rodriguez M., Reid M., Lustigman S. (2003). Clycophorin C is the receptor for the *Plasmodium falciparum* erythrocyte binding ligand PfEBP-2 (baebl). *Blood*, **101**, 4628-4631.

Matteelli A., Caligaris S., Castelli F., Carosi G. (1997). The placenta and malaria. *Annals of Tropical Medicine and Parasitology*, **91** (7), 803-810.

Mathias D.K., Pastrana-Mena R., Ranucci E., Tao D., Ferruti P., Ortega C., Staples G.O., Zaia J., Takashima E., Tsuboi T., Borg N.A., Verotta L., Dinglasan R.R. (2013) A small molecule glycosaminoglycan mimetic blocks *Plasmodium* invasion of the mosquito midgut. *PLOS Pathogens* **9** (11), e1003757. doi:10.1371/journal.ppat.1003757.

Melo M. R. S., Feitosa J. P. A., Freitas A. L. P., de Paula R. C. M. (2002). Isolation and characterization of soluble sulfated polysaccharide from *Gracilaria cornea*. *Carbohydrate Polymers*, **49**, 491-498.

Miyamoto K., Asakawa Y., Arai Y., Shimizu T., Tokita M., Komai T. (2001). Preparation of gellan sulfate as an artificial ligand for removal of extra domain A containing fibronectin. *Int. J. of Biol. Macromolecules*, **28**, 381-385.

Miyamoto K., Sato I., Tsutsui M., Uchino M., Takasaki S., Takebayashi T., Shimizu Y., Nobori T., Abe Y., Horiuchi T. (2010). Gellan sulfate selectively suppresses the activation of hemocoagulation factors XI and XII. *Materials Science and Engineering C*, **30**, 364-368.

Muschin T., Kanamoto T., Nakashima H., Yoshida T. (2012). Synthesis and potent biological activities of sulfated galactomannans. *Poster presentation: The 9th SPSJ International Polymer Conference* (IPC2012).

Nacer A., Movila A., Baer K., Mikolajczak S. A., Kappe S. H. I., Frevert U. (2012). Neuroimmunological blood brain barrier opening in experimental cerebral malaria. *PLoS Pathogens,* **8** (10): e1002982. doi:10.1371/journal.ppat.1002982.

Neill L., Hunt N. H. (1992). Pathology of fatal and resolving *Plasmodium berghei* cerebral malaria in mice. (abstract) *Parasitology,* **105** (2), 165-175.

Ogaji I. J., Nep E. I., Audu-Peter J. D. (2012). Advances in natural polymers as pharmaceutical excipients. *Pharmaceutica Analytica Acta* , **3**, 146.

Ono L., Wollinger W., Rocco I. M., Coimbra T. L. M., Gorin P. A. J., Sierakowski M. R. (2003). *In vitro* and *in vivo* antiviral properties of sulfated galactomannans against yellow fever virus (BeH111 strain) and dengue 1 virus (Hawaii strain). *Antiviral Research*, **60**, 201-208.

Persson K. E. M, Lee C. T., Marsh K., Beeson J. G. (2006). Development and optimization of high-throughput method to measure *Plasmodium falciparum*-specific growth inhibitory antibodies. *Journal of Clinical Microbiology,* **44**, 1665-1673.

Ponsford M. J., Medana I. M., Prapansilp P., Hien T. T., Lee S. J., Dondorp A. M., Esiri M. M., Day N. P., White N. J., Turner G. D. (2012). Sequestration and microvascular congestion are associated with coma in human cerebral malaria. *The Journal of Infectious Diseases,* **205** (4), 663-671.

Radfar A., Mendez D., Moneriz C., Linares M., Martin-Garcia P., Puyet A., Diez A., Bautista J. M. (2009). Synchronous culture of *Plasmodium falciparum* at high parasitemia levels. *Nature Protocols,* **4,** 1899 – 1915.

Ramos T. N., Bullard D. C., Darley M. M., McDonald K., Crawford D. F., Barnum S. R, (2013). Experimental cerebral malaria develops independently of endothelial expression of intercellular adhesion molecule-1 (ICAM-1). *The Journal of Biological Chemistry*. **288**: 10962-10966.

Rénia L., Howland S. W., Claser C., Charlotte Gruner A., Suwanarusk R., Hui Teo T., Russel B., Ng L. F. (2012). Mysteries at the blood-brain barrier. *Virulence,* **3** (2), 193–201.

Rinaudo M., Milas M. (2000). Gellan gum, a bacterial gelling polymer. *Novel Macromolecules in Food Systems – Developments in Food Science 41* Amsterdam: Elsevier Science B.V.

Rochas C., Lahaye M., Yaphe W. (1986). Sulfate content of carrageenan and agar determined by infrared spectroscopy. *Botanica Marina*, **29**, 335-340.

Rudd T. R., Hughes A., Holman J., Solari V., Ferreira E. O., Domingues R. M. C. P., Yates E. A. (2012). Heparan sulphate, its derivatives and analagoues share structural characteristics that can be exploited, particulary in inhibiting microbial attachment. *Brazilian Journal of Medical and Biological Research,* **45**, 386-391.

Samarasekera, U. (2009). Countries race to contain resistance to key antimalarial. *The Lancet*, **374**, 277-280.

Schmidt K. E., Schumak B., Specht S., Dubben B., Limmer A., Hoerauf A. (2011). Induction of pro-inflammatory mediators in *Plasmodium berghei* infected BALB/c mice breaks blood-brain-barrier and leads to cerebral malaria in an IL-12 dependent manner. *Microbes and Infection*, **13** (10), 828-836.

Schwartz L., Brown G. V., Genton B., Moorthy V. S. (2012). A review of malaria vaccine clinical projects based on the WHO rainbow table. *Malaria Journal*, **11**, doi:10.1186/1475-2875-11-11.

Shah J. N., Jani G. K., Parikh J. R. (2007). Gellan gum and its applications - a review. *Pharmaceutical Information, Articles and Blogs*, **5,** http://www.pharmainfo.net/reviews/gellan-gum-and-its-application---review.

Shih S., Wang Z., Guo S., Li L. (2007). Anticoagulant activity of cellulose sulfates with different intrinsic viscosities. *Asian Journal of Pharmaceutical Sciences*, **2**, 38-43.

Silva F. R. F,. Dore C. M. P. G., Marques C. T., Nascimento M. S., Benevides N. M. B., Rocha H. A. O., Chavante S. F., Leite E. L. (2010). Anticoagulant activity, paw edema and pleurisy induced carrageenan: Action of major types of commercial carrageenans. *Carbohydrate Polymers*, **79**, 26–33.

Spadafora C., Gerena, L., Kopydlowski K. M. (2011). Comparison of the *in vitro* invasive capabilities of *Plasmodium falciparum* schizonts isolated by Percoll gradient or using magnetic beads separation. *Malaria Journal*, **10**, doi:10.1186/1475-2875-10-96.

Sullivan 2012. Cinchona alkaloids: quinine and quinidine. In: *Treatment and Prevention of Malaria* Staines, H.M. and Krishna, S. (eds.). (pp. 45-67). AG: Springer Basel.

Tang F., Chen F., Li F. (2013). Preparation and potential *in vivo* anti-influenza virus activity of low molecular-weight κ-carrageenans and their derivatives. *Journal of Applied Polymer Science,* **127**, 2110-2115.

Taylor-Robinson A. W. (2010). Validity of modelling cerebral malaria in mice: argument and counter argument. *Journal of Neuroparasitology*, **1**, Article ID N100601, 5 pages doi:10.4303/jnp/N100601.

Thanh, T. T. T., Yasunaga, H., Takano, R., Urakawa, H., Kajiwara, K. (2001). Molecular characteristics and gelling properties of carrageenan family - 2. Tri-sulfated and tetra-sulfated carrageenans. *Polymer Bulletin* **47** (3-4), 305-312.

Thomson A.W. and Horne C.H. (1976). Toxiity of various carrageenans in the mouse. *The British Journal of Experimnetal Pathology* **57** (4), 455-459.

van de Velde, F., Knutsen, S. H., Usov, A. I., Rollema, H. S., Cerezo, A. S. (2002). H-1 and c-13 high resolution nmr spectroscopy of carrageenans: Application in research and industry. *Trends in Food Science & Technology* **13** (3), 73-92.

van de Velde, F., Pereira, L. and Rollema, H.S. (2004). The revised NMR chemical shift data of carrageenans. *Carbohydrate Research* **339** (13), 2309-2313.

van der Heyde H. C, Nolan J., Combes V., Gramaglia I., Grau G. E. (2006). A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. *Trends in Parasitology*, **22** (11), 503-508.

Vlieghe P., Clerc T., Pannecougue C., Witvrouw M., De Clercq E., Salles J.-P., Kraus J.-L. (2002). Synthesis of new covalently bound κ-carrageenan-AZT conjugates with improved anti-HIV activities. *Journal of Medicinal Chemistry,* **45**, 1275-1283.

Vogt A. M., Pettersson F., Moll K., Jonsson C., Normark J., Ribacke U., Egwang T. G., Ekre H. P., Spillmann D., Chen Q., Wahgren M. (2006). Release of sequestered malaria parasites upon injection of a glycosaminoglycan. *PLoS Pathogens,* **2** (9): e100, 0853-0863. doi:10.1371/journal.ppat.0020100.

Wang F. F., Yao Z., Wu H. G., Zhang S. X., Zhu N. N., Gai X. (2011). Antibacterial activities of kappa-carrageenan oligosaccharides (abstract). *Mechanical Engineering and Materials Science*, **108**, 194-199.

Wang W., Zhang P., Hao C., Zhang X. E., Cui Z. Q., Guan H. S. (2011). *In vitro* inhibitory effect of carrageenan oligosaccharide on influenza A H1N1 virus. *Antiviral Research*, **92,** 237-246.

White N. J. (1998). Malaria pathophysiology. In: Malaria: parasite biology, pathogenesis and protection. Sherman IW (Ed.), ASM Press.

Wilson D. W., Langer C., Goodman C. D., McFadden G. I., Beeson J. G. (2013). Defining the timing of action of antimalarial drugs against *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy*, **57**, 1455-1467.

World Health Organization. (2010). WHO: *Guidelines for the Treatment of Malaria - 2nd edition* Geneva: WHO Press.

World Health Organization. (2012). World Malaria Report 2012. Geneva, WHO.

Xiao L., Yang C., Patterson P. S., Udhayakumar V., Lal A. A. (1996). Sulfated polyanions inhibit invasion of erythrocytes by *Plasmodium* merozoites and cytoadherence of endothelial cells to parasitized erythrocytes. *Infection and Immunity,* **64**, 1373-1378.

Yahata K., Treeck M., Culleton R., Gilberger T. W., Kaneko O. (2012). Time-lapse imaging of red blood cell invasion by the rodent malaria parasite *Plasmodium yoelii*. *PLOS One*, **7** (12): e50780. doi:10.1371/journal.pone.0050780.

Yuan H., Song J., Li X., Li N., Dai, J. (2006). Immunomodulation and antitumor activity of κ-carrageenan oligosaccharides. *Cancer Letters*, **243**, 228-234.

Yuan H., Song J., Li X., Li N., Liu S. (2011). Enhanced immunostimulatory and antitumor activity of diferent derivatives of κ-carrageenan oligosaccharides from *Kappaphycus striatum*. *Journal of Applied Phycology,* **23**, 59-65.

Yuan H., Zhang W., Li X., Lu X., Li N., Gao X., Song J. (2005). Preparation and *in vitro* antioxidant activity of κ-carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives. *Carbohydrate Research,* **340**,685-692.

Yuan, H.M., Zhang, W.W., Li, X. G., Lu, X. X., Li, N., Gao, X. L. and Song, J.M. (2005). Preparation and *in vitro* antioxidant activity of kappa-carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives. *Carbohydrate Research,* **340** (4), 685-692.

Zhang, Y., Jiang N., Lu H., Hou N., Piao X., Cai P., Yin J., Wahlgren M., Chen Q. (2013).

Proteomic analysis of *Plasmodium falciparum* schizonts reveals heparin-binding merozoite proteins. *Journal of Proteome Research*, **12**, 2185-93.

LIST OF PUBLICATIONS

Iwanaga T., Sugi T., Kobayashi K., Takemae H., Gong H., Ishiwa A., Murakoshi F., **Recuenco F.C.**, Horimoto T., Akashi H., Kato K. Characterization of *Plasmodium falciparum* cdc2-related kinase and the effects of a CDK inhibitor on the parasites in erythrocytic shizogony. **Parasitol. Int**. (2013) 62 (5)

Kurokawa H., Kato K., Iwanaga T., Sugi T., Sudo A., Kobayashi K., Gong H., Takemae H., **Recuenco F.C**., Horimoto T., and Akashi H. "Identification of *Toxoplasma gondii* cAMP Dependent Protein Kinase and its Role in Tachyzoite Growth." **PLOS One** (2011) 6 (7).

Sugi T., Kato K.,Kobayashi K., Kurokawa H., Takemae H.,Gong H., **Recuenco F.C.**., Iwanaga T., Horimoto T.,Akashi H.."1NM-PP1 treatment of mice infected with *Toxoplasma gondi*." **J. Vet. Med. Sci.** (2011) 73 (10) : 1377.

Kobayashi K, Takano R, Takemae H, Sugi T, Ishiwa A, Gong H, **Recuenco FC**, Iwanaga T, Horimoto T, Akashi H , Kato K. Analyses of Interactions Between Heparin and the Apical Surface Proteinsof *Plasmodium falcipa rum.* **Scientific Reports** 3, doi:10.1038/srep03178.

Takemae H, Sugi T, Kobayashi K, Gong H, Ishiwa A, **Recuenco FC**, Murakoshi F, Iwanaga T, Inomata A, Horimoto T, Akashi H, Kato K. Characterization of the interaction between*Toxoplasma gondii* rhoptry neck protein 4 and host cellular β-tubulin. **Scientific Reports** 3**,** doi:10.1038/srep03199.

SUMMARY

論 文 の 内 容 の 要 旨

獣医学　専　攻
平成 22 年度博士課程 入学
氏　　名 ﾚｸｴﾝｺﾌﾗﾝｾｽｶｶﾞﾔｯﾄ
指導教員名　堀本泰介

論文題目　**Studies on the effects of sulfated polysaccharides on malaria infection**

（マラリア感染への硫酸化多糖類の効果に関する研究）

Malaria is a global concern. Concerted efforts have been geared to eradicate malaria especially in endemic areas, which are mostly developing countries. Vaccines that can prevent malaria infection are currently under testing while the screening for new antimalarial drugs is also a continuing process. Most of the antimalarial drugs that are currently being used during the blood stage of malaria when the *Plasmodium* parasites have already invaded red blood cells (RBCs) and the clinical symptoms of disease, including fever, are already presented. So there is a current interest in developing vaccines and drugs that can prevent the invasion of RBCs, so that the infection is halted altogether. Heparin is a sulfated glycosaminoglycan that can inhibit the entry of the *Plasmodium* parasites into the RBCs. However, it is not recommended for use in clinical malaria for its anticoagulant effects. Other sulfated polysaccharides like carrageenans from seaweeds were shown to have the same inhibitory effect on *Plasmodium* parasites *in vitro*. But their effect *in vivo* are not yet extensively studied. This work explored a novel anticoagulant, gellan sulfate, derived from the microbial polysaccharide,gellan gum, and other derivatives of carrageenans on their effects on the growth and invasion of malaria parasites *in vitro* and *in vivo*.

Gellan sulfate and oversulfated κ-carrageenan were prepared by adding DMF-SO_3 to gellan gum. λ-Carrageenan was modified by acid hydrolysis. The sulfation of gellan and κ-carrageenan were determined by

nuclear magnetic resonance spectroscopy and the level of sulfation were measured by elemental analysis.

Gellan sulfate, but not oversulfated κ-carrageenan and hydrolyzed λ-carrageenan, nor the native gellan gum, was shown to inhibit the growth and invasion of RBCs by *P. falciparum* in *in vitro* inhibition assays. Gellan sulfate was also shown to have low cytotoxicity and anticoagulant effects and thus, it was further assessed in *in vivo* studies using rodent malaria models.

In the 4-day suppressive test of growth *in vivo*, BALB/c and C57BL/6 mice were infected with the lethal parasites, *P. yoelii* 17XL and *P. berghei* ANKA, respectively. Gellan sulfate and native gellan gum were tested against artesunate (20mg/kg), an artemisinin derivative which is one of the recommended treatments for severe malaria. With various tested doses, of 20, 25, and 50mg/kg given to mice intraperitoneally, gellan sulfate and native gellan gum were found to be ineffective in inhibiting growth of the parasites *in vivo*. The parasitemias continued to rise after suppressive treatment and the mice eventually succumbed to the disease from 6 to 30 days post-infection. In addition, at 50mg/kg, gellan gum and gellan sulfate treated mice were observed to have weight loss and ruffled hair. This might indicate that at this dose, gellan gum and gellan sulfate were toxic to the mice.

Carrageenans have been shown to inhibit the growth and invasion of RBCs by *P. falciparum in vitro*. However, λ-carrageenan was also shown to increase the permeability of the blood brain barrier when administered to rats. Therefore, the use of carrageenans in malaria may actually cause cerebral malaria (CM). In this work, I examined the effect of carrageenans on the development of CM in the BALB/c mouse, considered as resistant strain, using *P. berghei* ANKA, a standard model for experimental CM. It is found that λ-carrageenan (25mg/kg) can induce symptoms and histopathological lesions related to CM.