IMMUNE RESPONSE TO INFECTION

Protective activity of *Borrelia duttonii*-specific immunoglobulin subclasses in mice

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To analyse the immune response of mice to *Borrelia duttonii* infection, BALB/c mice were inoculated intraperitoneally with *B. duttonii* strain 406K, and the titres of *B. duttonii*-specific immunoglobulins – IgM, IgG1, IgG2a, IgG2b and IgG3 – were determined by ELISA. IgM antibodies appeared first, followed by IgG2a and IgG3, and then IgG1 and IgG2b. The protective activity of individual classes and subclasses of *B. duttonii*-specific immunoglobulins was then determined by passive immunisation of BALB/c mice with immunoglobulin preparations purified by affinity chromatography. The mice were then challenged by intraperitoneal inoculation of *B. duttonii*. The study demonstrated that *B. duttonii*-specific IgM and IgG3 protected against the development of spirochaetaemia and death after borrelial infection, whereas *B. duttonii*-specific IgG1, IgG2a and IgG2b had low protective activities.

Introduction

*Borrelia duttonii*, the cause of tick-borne human relapsing fever, is still prevalent in Africa [1]. Man becomes infected when bitten by the infected vector, *Ornithodoros moubata*. The organisms multiply and cause spirochaetaemia within a few days after entering the human body. They are then cleared by antibody-mediated immunity. However, a few spirochaetes are able to escape from the immune response by antigenic variation and then multiply, resulting in a relapse of spirochaetaemia. As the humoral system plays the most important role in protection against borrelia, this infection appears to offer a suitable system for analysis of not only the immune response, but also the protective activity of pathogen-specific immunoglobulins (Ig).

It has been reported that IgG class immunoglobulins in mice and rats are transferred from mother to young via the fetal yolk sac and postnatal intestine through milk and colostrum, and that certain IgG isotypes are selectively transported via either of these routes [2, 3]. If this is the case, the protective role of each IgG isotype might be determined by the immune status of the offspring born or fed by mothers that relapse into fever just before or during pregnancy. An earlier study analysed the transfer of *B. duttonii*-specific IgG isotypes in ddY mice by cross-feeding and demonstrated that anti-borrelial IgG3 has a strong protective activity, and that IgG1 and IgG2a have some protective activity [4]. However, it could not determine the protective activity of IgG1 and IgG2a separately, nor could it determine the activity of IgG2b which is not transferred to the offspring.

This study investigated the immune response of an inbred mouse strain, BALB/c, and defined the protective role of individual classes and subclasses of *B. duttonii*-specific immunoglobulins by passive immunisation with purified immunoglobulin preparations.

Materials and methods

Organisms

*B. duttonii* strain 406K has been maintained in this laboratory for >15 years by passage in ddY mice (closed colony; Seiwa Experimental Animals Ltd, Fukuoka, Japan) at intervals of 3 days. One drop of blood was obtained from each infected mouse by cutting the tip of the tail, and diluted in 0.5 ml of 10 mM sodium phosphate-buffered saline (PBS), pH 7.4, containing sodium citrate 0.4%. Then, 0.2 ml of the diluted sample containing $10^5$–$10^6$ borreliae was injected intraperitoneally into mice to transmit the infection.

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Animals
A total of 106 BALB/c female mice (6–8 weeks old) were purchased from Seac Yoshitomi Ltd (Yoshitomi, Kyushu, Japan) and maintained under specific pathogen-free conditions in the Institute of Laboratory Animals in the medical school (temperature, 22°C ± 2°C, humidity 60 ± 10%, 12 h light/12 h dark cycle). Animals were given antibiotic-free, sterilised food and sterilised water ad libitum. This experiment was reviewed by the Committee of Ethics on Animal Experiment in the Yamaguchi University School of Medicine and conducted under the control of the Guidelines for Animal Experiments in Yamaguchi University School of Medicine and the Law and Notification of the Government.

Determination of spirochaetaemia
BALB/c mice were inoculated intraperitoneally with 0.2 ml of a suspension containing 100 viable borreliae as described previously [4]. Spirochaetaemia was determined daily by taking one drop of blood from the cut tail tip, mounting it on a glass slide and observing by dark-field microscopy with a ×40 objective and a ×10 eyepiece. Samples with no borrelia in 50 optical fields were regarded as negative, and those with >20 borreliae in one optical field were regarded as having strong spirochaetaemia.

Determination of titres of B. duttonii antibody
The titres of anti-B. duttonii antibodies in mouse plasma and purified isotype fractions were determined with an enzyme-linked immunosorbent assay (ELISA) as described previously [4]. Briefly, microtitration wells were coated with 0.1 ml of 5 μg/ml sonicates of B. duttonii grown in vitro [5], blocked with 0.2 ml of 0.1 M sodium phosphate buffer (PB), pH 7.5, containing bovine serum albumin 1%, and added to 0.1 ml of plasma and purified isotype fractions were determined from 18 infected mice 3 months after inoculation. Plasma samples (7.2 ml), with anti-IgG1, -IgG2a, -IgG2b and -IgG3 conjugates, <0.4% with the anti-IgG2a conjugate and <0.4% with the anti-IgG1 conjugate. The latter showed 1.6% cross-reactivity with IgG2a.

Purification of immunoglobulins
For the purification of IgM, blood was collected from three infected mice by puncturing the heart 7–10 days after inoculation with B. duttonii. Blood cells were removed, and the resulting plasma (1.3 ml) with an IgM titre of 5120 was applied to a Sepharose CL-6B column (Pharmacia Fine Chemicals, Uppsala, Sweden) with a bed volume of 270 ml, equilibrated with PB, pH 7.5, and eluted with the same buffer. The absorbance at 280 nm and the ELISA titres of anti-B. duttonii antibodies in 2-ml fractions were measured.

For purification of IgG subclasses, blood was collected from 18 infected mice 3 months after inoculation. Plasma samples (7.2 ml), with anti-IgG1, -IgG2a, -IgG2b and -IgG3 titres of 163840, 20480, 327680 and 81920, respectively, were diluted five-fold with PB, pH 8.0, and applied to a Protein-A Sepharose CL-4B column (Pharmacia Fine Chemicals) with a bed volume of 8 ml equilibrated with PB, pH 8.0. After washing with the same buffer until the optical density at 280 nm was <0.01, IgG1 molecules were eluted with 60 ml of 0.1 M citrate buffer, pH 6.0. Then the column was developed with a gradient of pH 6.0–3.5 in 0.1 M citrate buffer (60 ml + 60 ml) followed by 60 ml of 0.1 M citrate buffer, pH 3.5. The absorbance at 280 nm of each fraction (2 ml) was measured, and the fractions were neutralised to pH 7.0 with 1 M Tris-Cl buffer, pH 8.5. After the antibody titres had been measured by ELISA, the appropriate fractions were pooled and concentrated with a Centricon-30 concentrator (Amicon Corp., Danvers, MA, USA). The resulting IgG subclass preparations were further purified by passing them through a Sepharose CL-4B affinity column coupled with contaminating rabbit anti-mouse IgG subclasses. The final preparations were concentrated and dialysed against saline at 4°C.

Passive immunisation studies
Mice were immunised passively with purified isotype preparations as follows: BALB/c mice were inoculated subcutaneously with 0.2 ml of each isotype solution, and 6 h later, inoculated intraperitoneally with 0.2 ml of a B. duttonii suspension containing c. 100 viable micro-organisms. The highest protein concentrations (mg/ml) injected were 0.21, 0.016, 0.05, 0.025 and 0.011 for IgM, IgG1, IgG2a, IgG2b and IgG3, respectively. The control mice were inoculated subcutaneously with the same volume of pooled normal mouse serum, and similarly inoculated with B. duttonii. After the challenge infection, the course of spirochae-
The course of spirochaetaemia and the antibody responses of BALB/c mice inoculated with *B. duttonii*

The course of spirochaetaemia and antibody responses were investigated in five BALB/c mice after intraperitoneal inoculation of 100 borreliae. Borreliae appeared in the blood 2–3 days after injection and then increased in number. All five mice showed strong spirochaetaemia from the fifth to eighth day of infection (Fig. 1). Three mice died on day 8, but two mice survived and completely cleared spirochaetes from the blood after showing a few relapses.

IgM was detected 4–6 days after inoculation, peaked from day 8 to day 10, and gradually decreased in the surviving mice. IgG subclasses were detected only in the surviving mice; IgG2a and IgG3 appeared on days 7–8 followed by IgG1 and IgG2b on days 12–15.

**Purification of *B. duttonii*-specific immunoglobulins**

*B. duttonii*-specific IgM was purified by gel filtration chromatography from the plasma of mice 7–10 days after infection. The purified preparation was essentially free from *B. duttonii*-specific IgG subclasses (Table 1). *B. duttonii*-specific IgG isotypes were purified by

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**Table 1. Immunoglobulin (Ig) preparations purified from immunised mice**

<table>
<thead>
<tr>
<th>Ig preparation</th>
<th>IgM</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
<th>IgG3</th>
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<tbody>
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<td>IgM</td>
<td>1280</td>
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<td>&lt; 20</td>
<td>&lt; 20</td>
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<td>IgG1</td>
<td>80</td>
<td>81920</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>320</td>
</tr>
<tr>
<td>IgG2a</td>
<td>20</td>
<td>&lt; 20</td>
<td>2560</td>
<td>&lt; 20</td>
<td>80</td>
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<tr>
<td>IgG2b</td>
<td>&lt; 20</td>
<td>20</td>
<td>80</td>
<td>5120</td>
<td>160</td>
</tr>
<tr>
<td>IgG3</td>
<td>&lt; 20</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>2560</td>
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</tbody>
</table>

*1 in 20 was the lowest dilution tested.
affinity chromatography from the pooled plasma collected 3 months after infection. It should be noted that the final IgG preparation still contained a small amount of the other IgG isotypes (Table 1).

**Passive immunisation of mice with purified immunoglobulins**

The results of the passive immunisation studies for the purified classes or subclasses of immunoglobulins are summarised in Table 2. Seventeen of 18 control mice given pooled normal mouse serum exhibited strong spirochaetaemia when inoculated with borreliae and 10 mice died.

Mice given the IgM preparation that had an ELISA titre of 320 were fully protected from borrelial inoculation; none of the six mice in this group showed any grade of spirochaetaemia. Dose-dependent protection from spirochaetaemia was observed in mice given an IgM preparation with an ELISA titre of 32 and 3.2. Mice given an IgM preparation with an ELISA titre of 0.32 were not protected at all from spirochaetaemia, but did not die.

When the mice were given each of the IgG subclass preparations, those with an IgG3 titre of 320, and IgG1 and IgG2b titre of 3200 gave complete protection. Similarly, injection of an IgG2a preparation with a titre of 2560 resulted in almost full protection. However, IgG2a preparations with a titre of 3.2, IgG1 with a titre of 32 and IgG2b with a titre of 320 did not have any protective effect on spirochaetaemia, but had some protective effects against lethal infection. An IgG3 preparation with titre of 3.2 had no protective effect on spirochaetaemia and death occurred after the borrelial challenge. These results indicate that anti-borrelial IgG3 has a stronger protective activity than other IgG isotypes. Taking into account the contaminating IgG3 titres in the IgG preparations (IgG1, 1 in 256; IgG2a, 1 in 32; IgG2b, 1 in 32), anti-borrelial IgG1, IgG2a and IgG2b have low but significant protective activity against spirochaetaemia and death.

**Discussion**

The genus *Borrelia* contains three major groups of organisms that are pathogenic for man or other animals: (i) several species that cause louse-borne and tick-borne relapsing fever; (ii) *B. burgdorferi* and related species – the aetiological agents of Lyme arthritis, erythema migrans, tick-borne meningoradiculitis and acrodermatitis chronica atrophicans; and (iii) *B. anserina*, the cause of avian spirochaetosis. Many authors have investigated humoral responses and passive immunisation against *B. burgdorferi*. In contrast, few studies have been done on *B. duttonii*, the causative agent of relapsing fever. A previous study reported the in-vitro cultivation of *B. duttonii* [5] and developed an ELISA with *B. duttonii* cultivated *in vitro* as the antigen, which made it possible to investigate specific antibodies in mouse blood over the course of *B. duttonii* infection.

When *B. duttonii* was injected into BALB/c mice, IgM appeared first, followed by the IgG subclasses, of which IgG2a and IgG3 appeared earlier than IgG1 and IgG2b. The results were different from the previous observations in ddY mice, i.e., IgG2b and IgG3 appeared earlier than IgG1 and IgG2a [4]. The difference in the IgG2a and IgG2b responses may be due to the difference between mouse strains [6–8]. In the case of *B. burgdorferi* infection in BALB/c mice, IgM was detected 5 days after infection, followed by

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**Table 2** Development of spirochaetaemia and death of BALB/c mice after challenge with *B. duttonii* after passive immunisation

<table>
<thead>
<tr>
<th>Ig preparation</th>
<th>Antibody titre</th>
<th>Number of mice tested</th>
<th>Number with spirochaetaemia (number dead) days after infection</th>
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<tbody>
<tr>
<td></td>
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<td>Total 1 2 3 4 5 6 7 8 9</td>
<td></td>
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<tr>
<td>IgM</td>
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<td>32</td>
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<td>3200</td>
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<td>IgG2a</td>
<td>2560</td>
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<td>1 (0) 1 (0) 1 (0) 1 (0) 1 (0) 1 (0) 1 (0) 1 (0) 1 (0)</td>
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<tr>
<td>IgG2b</td>
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<tr>
<td>IgG3</td>
<td>320</td>
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<td>control</td>
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<td>18</td>
<td>17* (10) 17* (10) 17* (10) 17* (10) 17* (10) 17* (10) 17* (10) 17* (10) 17* (10)</td>
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* All the mice showed strong spirochaetaemia (see Materials and methods).
simultaneous IgG1, IgG2a and IgG3 responses on day 8 [9].

It is interesting that IgG3 is one of the earlier responsive IgG subclasses during B. duttonii infection in both BALB/c and ddY mice. Yang et al. [10] reported that in the case of B. burgdorferi infection, BALB/c mice displayed primarily a Th2 phenotype response which may induce IgG3 synthesis [11]. Although there is no direct evidence of which T helper subset, Th1 or Th2, predominates over the other in B. duttonii infection, the Th2 immune response might be dominant and cause IgG3 responses earlier than those of other subclasses.

Arimitsu et al. [12] reported that passively transferred IgM antibodies can protect animals from B. duttonii infection. However, little is known about which of the IgG isotypes plays an important role in protection against B. duttonii infection. The passive protective activity of monoclonal IgG3 against B. burgdorferi has been reported [13], but there have been no studies with purified IgG subclasses from infected mice that compared the activities between isotypes. This study demonstrated that B. duttonii-specific IgM and IgG3 have a strong protective activity, and B. duttonii-specific IgG1 and IgG2a have a weak protective activity against the development of spirochaetaemia and death after borrelial challenge infection. These results coincided well with previous observations in ddY mice [4]. In addition, the present study showed that B. duttonii-specific IgG2b protects against death but not against the development of spirochaetaemia.

There are two major mechanisms for bacterial clearance. One is opsonisation-phagocytosis, and the other is complement-mediated lysis. All the immunoglobulin isotypes except IgG1 activate complement via the classical pathway, which mediates phagocytosis by binding to the complement receptor on polymorphonuclear leucocytes. Conversely, all the IgG isotypes, but not IgM, have specific Fc receptors on murine macrophages which mediate phagocytosis [14]. Thus IgG2a, IgG2b and IgG3 may contribute to both complement-dependent and independent bacterial clearance, whereas IgG1 and IgM may contribute only to the complement-independent pathway.

The results of passive immunisation against B. duttonii show that IgG3 is much more protective than the other IgG isotypes. A previous study reported that IgG3 transferred from mother to young through the placenta completely protected against borrelial infection in ddY mice [4]. Similar findings that IgG3 has a stronger protective activity than other IgG isotypes in infections with Trypanosoma gambiense [15], Vibrio cholerae El Tor [16], Plasmodium vivax [17], P. yoelii [18–20] and Streptococcus pneumoniae [21] have been reported. It was reported that not only IgG3 but also IgG2a and IgG2b activate complement [22]. Therefore, complement-mediated clearance alone could not explain the difference in the protective efficacy of these three isotypes. It is possible that the binding efficacy of IgG isotypes to their Fc receptors differs. If the binding of IgG3 is higher than that of the other IgG isotypes, borreliae opsonised with specific IgG3 may be captured more efficiently by phagocytes through the IgG3 Fc receptor, thus leading to effective phagocytosis.

It is well known that B. duttonii achieves a degree of immortality through multiphasic antigenic variation in its major outer-membrane proteins by responding to the adaptable immune responses of the host. Further studies including identification of epitope(s) correlated with protection will be necessary to define whether the protective antibody is related to the specific immunoglobulin isotypes. It should be noted that this ELISA system may detect both protective and non-protective antibodies, as whole cell sonicates of cells grown in vitro were used as the antigen.

In summary, this study showed that anti-B. duttonii-specific IgM and IgG isotypes are capable of protecting mice against B. duttonii infection by passive immunisation. Among the IgG isotypes, IgG3 provides protective immunity, whereas the other isotypes do not.

References