Immunoglobulin subclass responses of wild brown rats to
*Sarcocystis singaporensis*

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Abstract

Immunoglobulin subclass responses of wild brown rats (*Rattus norvegicus*) from southeastern Asia to the endemic cyst-forming coccidian *Sarcocystis singaporensis* were characterised. The antibody response of brown rats to wild-type parasites (high reproductive capacity) showed a Th1 profile during acute infection, namely elevated concentrations of parasite-specific IgG2b and IgG2c and absence of IgG1. Chronic infection (bradyzoite development) resulted in a mixed Th1/Th2 pattern whereby significant concentrations of IgG1 appeared. A primary infection with 1000 sporocysts eight days before challenge induced protection, accompanied by significant concentrations of IgA and IgG2, particularly IgG2a. Western blot analysis of rat sera, using sporozoite and bradyzoite-extracts as antigen, revealed that IgG1, IgG2a, and IgG2b predominantly recognised molecules between 70–80 kDa in one or the other stage. Some of the antibodies were possibly directed against a 79 kDa heat shock protein of sporozoites. An apparent unresponsiveness to molecules in the low molecular weight range, particularly of bradyzoite antigens, was observed. This was abrogated by infection of rats with an avirulent strain of *S. singaporensis* (low reproductive capacity) indicating that a parasite that was less adapted to its host provoked a stronger immune response. These results suggest the existence of an immune evasion strategy used by *Sarcocystis* and show that wild rodents may be suitable as immunological research objects, reflecting the natural situation. © 2001 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Sarcocystis singaporensis*; *Rattus norvegicus*; Wild rats; Immunoglobulin subclass responses; Virulence

1. Introduction

Cyst-forming coccidia of the genus *Sarcocystis* are among the most prevalent parasites of livestock and responsible for considerable economic losses. They also infect many wild mammals, birds, cold-blooded animals, and humans (Dubey et al., 1989).

We became interested in immunity against *Sarcocystis* spp. when we studied the possible use of an endemic species, *Sarcocystis singaporensis*, as a biological control agent of wild rodent populations in southeastern Asia. Artificially high infection dosages induce high mortality among wild *Rattus* spp. due to schizogonic development in the lungs (Jäkel et al., 1999b) but can also immunise rats inducing resistance to subsequent infections (Jäkel et al., 1996). *Sarcocystis singaporensis* is widespread in southeastern Asia and prevalent in rodents as well as in the definitive hosts, certain boid snakes (O’Donoghue et al., 1987; Jäkel et al., 1997b). These observations suggest a high transmission success of the parasite. Data from our previous investigations indicate that this is probably due to its enormous proliferative capacity in both hosts. It seems to be well-adapted to wild rats because numbers of bradyzoites (the stage of the chronic phase of infection) residing in muscle tissue can be extremely high without inducing apparent signs of disease (Boonsong et al., 1999). In contrast, laboratory rat strains tolerate much lower numbers of parasites (Brehm and Frank, 1980). Although it is not known how many sporocysts (containing sporozoites, the stage infective for rats) are actually needed in the wild to induce a patent infection in a rat, we assume it to be quite few because natural infections apparently do not induce protective immunity (Boonsong et al., 1999). A significant immune response conferring protection can be induced experimentally using about 1000 sporocysts (Jäkel et al., 1996), a dosage that is well-tolerated by wild rats.
To learn more about specific immunity of the natural intermediate hosts, we measured the immunoglobulin subclass responses and their target antigens of experimentally infected wild brown rats, Rattus norvegicus, which were live-trapped in Thailand. It has been shown for rats that immunoglobulin subclasses can be indicative of the underlying Th1/Th2 pattern (Binder et al., 1995; Gracie and Bradley, 1996) as they are controlled and modulated by cytokines associated with the expansion of certain T helper cell subsets (Finkelman et al., 1988). It seems to be established that antibodies of the IgG2b and IgG2c subclasses can be assigned to a Th1 and IgG1 subclass antibodies to the Th2 T helper-dependent immune responses in the rat (Gracie and Bradley, 1996). Although some investigators regard IgG2a antibodies as a component of the Th2 response (Binder et al., 1995), accumulating evidence suggests that this subclass is upregulated by IFN-γ, thus, also indicative of a Th1 pathway (Uchikawa et al., 1994; Gracie and Bradley, 1996; Smith et al., 1997). To date, nothing is known about immunoglobulin subclass responses to Sarcocystis spp. Furthermore, knowledge of immune reactions of wild rodents to endemic pathogens is limited.

2. Materials and methods

2.1. Experimental animals

Adult wild brown rats (R. norvegicus) were live-trapped at Chachoengsao province, Thailand. This location was selected, because, for experimental purpose, we needed animals which were largely devoid of natural infections with Sarcocystis spp. A preliminary parasitological and serological examination (parasite-specific IgM and IgG) revealed that rodents collected in this particular area were rarely infected with Sarcocystis spp. (prevalence <2%, n = 211, T. JaÈkel, unpublished observation); other coccidia were not examined. Furthermore, the majority of rats actually used for the experiments reported here were found to be free of sarcocysts before 30 days post-infection (p.i.) (see section 3.1.), a time interval in which a natural infection is easily distinguished from the experimental one because appearance of sarcocysts usually needs a month (Boonsong et al., 1999). Finally, the serological results indicate that the animals were not naturally infected (for instance see IgM response).

Rats were caged individually in $50 \times 50 \times 26$ cm wire boxes equipped with removable sleeping trays. A diet consisting of commercially available pelleted cereals, vegetables, and water was offered. The rats were kept in the laboratory two weeks before the onset of the experiments. Unless otherwise indicated, experimental groups consisted of equal portions of males and females with a mean body mass in each group varying between 280–320 g. The rats were kept in an isolated room and received a standard diet and water ad libitum.

2.2. Parasites and infection

The strain of S. singaporensis used in most of the experiments, S5, was originally obtained from faeces of a reticulated python (Python reticulatus) captured in the wild in Thailand. This so-called ‘wild-type’ was passaged twice from rats to a python in the laboratory and has been characterised in detail elsewhere (Boonsong et al., 1999). Its main characteristics were a relatively high virulence ( = high capacity to produce bradyzoites in wild and laboratory rats, sensu Poulin and Combes, 1999) and an intermediate pathogenicity in wild rats. Sporocysts used for the experiments were 3-months-old and had been stored at 4°C.

A second, avirulent strain (S1, low capacity to form bradyzoites) was selected from the wild-type by 10 serial passages over a period of two years using Fischer rats (F-344) that, at each passage to a python, contained sarcocysts that were about 2–3 weeks old (high percentage of immature sarcocysts). This strain was completely apathogenic in wild brown rats regardless of the inoculation dose. Sporocysts of S1 were the same age as the wild-type and were stored accordingly.

For infection, wild rats were anaesthetized with methoxyflurane (Metofane, Janssen-Cilag, Germany) and subsequently inoculated with 400 μl of a sporocyst suspension in phosphate-buffered saline (PBS) using a stomach tube. Negative controls were treated likewise but received PBS only.

2.3. Parasite antigens

In the ELISA, a somatic extract of wild-type bradyzoites in PBS was used as antigen. Bradyzoites were obtained from sarcocysts in muscles of an experimentally infected laboratory rat (strain Wistar) by tryptic digestion as described by JaÈkel et al. (1999a). Then, the zoites were poured through a 100 μm nylon mesh. They were washed several times in Hank’s balanced salt solution (HBSS) by centrifugation at 400 x g. Approximately $2 \times 10^8$–4 x $10^8$ zoites were suspended in 1 ml PBS containing protease inhibitors ethylene diamine tetracetic acid disodium salt (EDTA, 1 mM), ethylene glycol bis-(2-amino ethyl) ether)-N,N,N′,N′-tetra- cetic acid (EGTA, 1 mM), N-ethylmaleimide (NEM, 1 mM), Pepstatin (1 μM), phenyl methyl sulphphonyl fluoride (PMSF, 1.1 μM), and N-tosylamide-L-phenylalanine chloromethyl ketone (TPCK, 100 μM) (all Sigma). Afterwards, bradyzoites were freeze-thawed four times and incubated on a shaker for 2 h at 4°C. Finally, the antigen was spun at 12,000 x g for 30 min at 4°C, the supernatant aspirated, and stored at $-30^\circ$C before use.

Western blot analysis was performed with Nonidet P40-extracts of sporocysts/sporozoites and bradyzoites. Approximately $1.3 \times 10^8$ sporocysts, purified by Percoll density-gradient centrifugation as described previously (JaÈkel et al., 1997a), were incubated in distilled water containing 8% NaOCl for 30 min at 20°C. Sporocysts
were washed three times in HBSS by centrifugation at 1600 \( \times \) g and the resulting pellet suspended in 1 ml lysis buffer (150 mM NaCl, 50 mM Tris-base, and 4 mM EDTA in distilled water, pH 8.0). Protease inhibitors were added and sporocysts were vortexed with glass beads (1 mm diameter) for 12 min at 20°C. The resulting suspension was aspirated and incubated in 2% Nonidet P40 on ice for 30 min. The extract was centrifuged at 12,000 \( \times \) g for 10 min and the supernatant stored in aliquots at \(-20°C\). Nonidet P40-extraction of bradyzoites followed the same protocol as described for the ELISA except that 2% Nonidet P40 was added to the lysis buffer after freeze-thawing of bradyzoites. Extracts of two parasite strains, S1 and S5, were prepared; the latter was used for all analyses except in experiment 4 where extracts of both strains were employed.

Protein concentrations of the antigen preparations were determined using the bicinchoninic acid (BCA) protein assay (Pierce). For analysis of Nonidet P40 extracts, the detergent was diluted to \(<1\%\) because higher concentrations are known to interfere with the BCA test procedure.

2.4. Antibodies

Alkaline phosphatase-conjugated polyclonal sheep-anti-rat immunoglobulin subclass antibodies (IgA, IgG1, IgG2a–c, IgM) for use in the ELISA were purchased from The Binding Site Ltd. Polyclonal anti-subclass antibodies for Western blotting were peroxidase-conjugated (IgG1, IgG2a, and IgG2b) or biotin-conjugated (IgA) (The Binding Site). Polyclonal rabbit antibodies prepared against purified heat shock protein 70 (hsp70) from E. coli (DnaK) were purchased from Upstate Biotechnology. DnaK, the bacterial homologue of mammalian hsp70, has more than 48% amino acid homology with mammalian hsp70s (Flaherty et al., 1990; Gething and Sambrook, 1992). A rabbit serum against bradyzoites of the wild-type strain of S. singaporensis was obtained as described previously (Jäkel et al., 1999a).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The ELISA was employed to detect parasite-specific immunoglobulin subclass responses of experimentally infected rats. Microtiter plates (96-well, Brand) were coated with 0.8 \( \mu \)g bradyzoite antigen/well in 0.1 M carbonate buffer (pH 9.6) for 12 h at 4°C. Plates were washed three times with 200 \( \mu \)l Tris-buffered saline (TBS, pH 7.4) containing 0.3% Tween 20 and 0.05% BSA (Biomol). Afterwards, 100 \( \mu \)l of the serum sample diluted in TBS was applied in each well and incubated for 2 h at 37°C. After washing three times with TBS, wells were incubated with 100 \( \mu \)l alkaline phosphatase-conjugated polyclonal sheep-anti-rat immunoglobulin subclass antibodies in TBS containing 0.05% BSA for 1 h at 37°C. Finally, wells were washed three times with TBS and 100 \( \mu \)l of a 1 M diethanolamine buffer containing 0.1% p-nitrophenyl-phosphate (Kirkegaard & Perry Lab. Inc.) were applied to each well and incubated for 30 min at 37°C. The enzyme reaction was stopped by addition of 100 \( \mu \)l EDTA (5% in distilled water). For each serum sample, duplicate measurements of absorbance at 405 nm were performed using a Dynex MRX microplate photometer (Dynatech Laboratories).

Suitable dilutions of serum samples and the corresponding alkaline phosphatase-conjugated antibodies had been determined by checkerboard-titration using sera of experimentally infected wild brown rats and negative controls reared under coccidia-free conditions in the laboratory. Sera were diluted 1:20 for all isotypes except IgG1 and IgM, which were used at 1:100 and 1:50, respectively. Alkaline phosphatase-conjugated antibodies were diluted 1:50 for detection of IgA, 1:300 for IgG1, 1:500 for IgG2a and IgG2c, 1:150 for IgG2b, and 1:1000 for IgM.

To allow for comparison among the various microtiter plates used in experiment 1, a reference-positive serum was run for each isotype on each plate as two-fold serial dilutions ranging from 1:100 to 1:12800. Those sera were selected as a reference that showed an O.D. between 0.9 and 1.1 at a dilution of 1:100. Titres of the serum samples were assessed using a standard serum titration curve and expressed as values relative to the standard serum (Crowther, 1995).

2.6. SDS Page and Western blotting

Nonidet-P40 extracts of S. singaporensis sporozoites/bradyzoites were solubilized in 0.125 M Tris-buffer, pH 6.8, containing 4% SDS, 20% glycerol, and 4% 2-mercaptoethanol, and heated for 3 min in boiling water. After centrifugation at 12,000 \( \times \) g, the supernatant was used for resolution by SDS PAGE (Laemmli, 1970). Proteins (30–35 \( \mu \)g per lane for each type of antigen) were separated at 25 mA constant current in 0.05 M Tris-buffer (pH 8.3) containing 0.38 M glycine and 0.1% SDS using 4% stacking gels and 12.5 or 14% resolving gels cast in a minigel chamber (BioRad). Electrical transfer onto 0.45 \( \mu \)m-pore-size nitrocellulose membranes (Schleicher and Schuell) was performed at 250 mA and 4°C in 25 mM Tris-buffer, pH 8.3, containing 192 mM glycine, 0.1% SDS, and 20% methanol for 1.5 h. All blots were blocked with 3% skimmed milk powder in PBS for 30 min at 20°C.

Strips of nitrocellulose membranes were incubated 1.5 h with rat sera diluted 1:30, 1:70, 1:70, 1:50, and 1:75 (for analysis of IgA, IgG, IgG1, IgG2a, and IgG2b, respectively) in PBS containing 1% skimmed milk powder. After washing in PBS supplemented with 0.05% Tween 20, strips were incubated with polyclonal peroxidase-conjugated anti-rat-immunoglobulin antibodies diluted 1:500 in PBS (anti-IgG, IgG1, IgG2a, and IgG2b, respectively) for 1.5 h. IgA antibodies were visualised with a biotin-conjugated anti-rat-IgA antibody followed by incubation with streptavidin-
peroxidase (Sigma) diluted 1:400 in PBS containing 1% skimmed milk powder. After washing, blots were developed with PBS containing 0.01% hydrogen peroxide, 0.02% 4-chloro-1-napthol, and 5.6% methanol.

Polyclonal rabbit anti-Dnak antibodies were used at a dilution of 4 mg/ml and incubated with blots for 12 h at 4°C. As control, rabbit antibodies prepared against Entamoeba invadens (Jakob and Jäkel, 1994) were incubated with nitrocellulose strips at a similar concentration. The rabbit serum prepared against wild-type bradyzoites of S. singaporensis was diluted 1:50 in PBS. All primary antibodies were detected with a goat-anti-rabbit IgG peroxidase-conjugate (Sigma) diluted 1:300 in PBS.

2.7. Experimental design and collection and storage of serum samples

Four infection experiments with wild brown rats were performed the design of which is summarised in Table 1. Blood samples were collected by puncture of the heart immediately after killing the animals with CO2. Serum samples were stored at −20°C before use.

2.8. Statistical analyses

Mean indices of antibody titres of the various animal groups of experiment 1 were analysed by a one way analysis of variance (ANOVA) after data had been normalised by logarithmic (log10) transformation. For multiple comparisons versus the control group, Tukey’s test was used. Because the data of experiments 2 and 3 were not normally distributed, experimental groups were compared by a Kruskal–Wallis one way analysis of variance on ranks. Multiple comparisons versus the control group were performed according to Dunn’s method.

3. Results

3.1. Kinetics of the antibody response after primary infection with S. singaporensis

There are two major parasitaemic events in rats infected with S. singaporensis: merozoite development in endothelial cells 6–25 days p.i. (acute phase) (Brehm and Frank, 1980; Jäkel et al., 1996) and cyst formation in the striated muscles which usually commences around 30 days p.i. (chronic phase) (Häfner, 1987). We plotted relative spleen mass against time p.i. and observed two distinct peaks which indicated immune responses to these two parasitemic events (Fig. 1). Throughout the experiment, rats showed no signs of illness. Examination of striated muscles revealed that small immature sarcocysts were present in most of the animals 32 days p.i. indicating a successful experimental infection. At later intervals, more than 90% of the animals contained mature sarcocysts in the muscles. None of the rats dissected before 32 days p.i. showed muscle cysts, confirming that these wild-caught animals were not chronically infected.

Analysis of the antibody response by ELISA revealed that similar to changes in the spleen, infection appeared to induce two peak antibody responses in rats. This was reflected, for instance, in the kinetics of the IgG2a and IgG2c response (Fig. 1). Wild brown rats showed a rapid antibody response after primary infection with high concentrations of IgM appearing 8 days p.i., concomitantly with parasite-specific IgA (Fig. 1). Significant concentrations of parasite-specific IgG2a, IgG2b, and IgG2c were first measured 24 days p.i., during the acute phase of infection, and were continuously present at later intervals. Significant concentrations of IgG1 were only seen during the chronic infection phase (Fig. 1).

To further confirm the observed pattern of immunoglobulin subclass responses (high concentrations of IgG2 but

<table>
<thead>
<tr>
<th>Infection experiment</th>
<th>Primary infection (No. of sporocysts)</th>
<th>Secondary challenge</th>
<th>No. of rats examined per group/day</th>
<th>Days of examination (d.p.i., or, days post challenge, d.p.c.)</th>
<th>Days of examination of uninfected controls</th>
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<tr>
<td>1 1000</td>
<td>1000</td>
<td>10</td>
<td>0, 8, 16, 24, 32, 60, 90 d.p.i. ³</td>
<td>16, 32, 90 d.p.i.</td>
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<tr>
<td>2</td>
<td>1000</td>
<td>10</td>
<td>18–22 d.p.i. &gt;60 d.p.i.</td>
<td>60 d.p.i.</td>
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<tr>
<td>3</td>
<td>1000</td>
<td>2 × 10⁷ sporocysts eight days after priming</td>
<td>12 (primed), 15 (challenge controls, seven males)</td>
<td>20–25 d.p.c., 11–20 d.p.c. ¹</td>
<td>(17 rats, nine males)</td>
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<tr>
<td>4</td>
<td>1000</td>
<td>4 females per parasite strain</td>
<td>57 d.p.i.</td>
<td>57 d.p.i.</td>
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³ For all experiments, parasite strain S5 was used, except for experiment 4, where antibody responses of rats to strains S1 and S5 were compared.

² Uninfected control rats were inoculated with PBS instead of parasites.

¹ Muscles of rats were extensively examined for presence of sarcocysts using a microscope.

º Rats were monitored constantly to obtain fresh blood samples when they died.

Table 1 Experimental design for infection of wild brown rats and examination of sera
no IgG1 antibodies during acute infection), we compared sera of a group of chronically infected wild brown rats with that of acutely infected animals (experiment 2). High concentrations of IgM were measured during the acute phase. Although IgA antibody levels of the acute phase were higher in single rats, the median of the group was not statistically different from the controls (Fig. 2). In contrast to the first experiment, no parasite-specific IgA was present during chronic infection. Similar to the previous results, IgG1 was not detectable during acute infection, whereas concentrations of parasite-specific IgG2b and IgG2c were significantly elevated when compared with the controls (Kruskal–Wallis ANOVA, d.f. = 2, Dunn’s multiple comparison versus control, $P < 0.05$). Sera of chronically infected brown rats showed high concentrations of parasite-specific IgG1, IgG2a, and IgG2c.

Fig. 1. Kinetics of the immunoglobulin subclass response in wild brown rats after primary infection with *S. singaporensis*. Serum samples were analysed by ELISA using bradyzoite-antigen prepared from the S5 strain. Antibody concentration is expressed as the reactivity relative to standard serum. Relative spleen masses were calculated as fresh spleen mass divided by body mass. Values are expressed as the mean ± SEM of 10 animals per time interval. Because experiments were conducted in Thailand where the parasite occurs in the wild, groups of 10 uninfected animals were examined at three different time intervals to control for a possible environmental contamination with sporocysts. For statistical analysis, groups of infected rats were compared with negative controls at similar time intervals. For instance, uninfected rats at day 16 were compared with infected rats at days 8 and 16.

Fig. 2. Immunoglobulin subclass responses of acutely (18–22 days p.i., $n = 10$) and chronically (>60 days p.i., $n = 10$) infected wild brown rats. Data are presented as dot-plots and bars indicate the median. Asterisks indicate significantly ($P < 0.05$) elevated antibody concentrations compared with the control ($n = 10$).
3.2. Antibody response following secondary challenge

Wild rats can mount a rapid protective immune response to a challenging infection with *S. singaporensis* if they are primarily infected (immunised) with a high but sublethal number of sporocysts. In protected rats, development of schizonts in endothelial cells of the lungs is inhibited and they therefore survive acute disease (Jäkel et al., 1996). We investigated if there is a difference in immunoglobulin subclass responses of immunised (8 days before challenge) versus non-immunised animals. All rats which did not receive sporocysts before challenge died 11–20 days p.i. due to extensive schizonic development. During this period, immunised rats also became moribund showing signs of respiratory disease, but they recovered and their sera were collected 20–25 days post challenge. As shown in Fig. 3, concentrations of all immunoglobulins, except IgG2a in non-immunised animals and IgG1, were significantly elevated in the infected groups compared with the controls. Thus, the main difference between the two infected groups was the absence of IgG2a in non-immunised rats.

3.3. Western blot analysis of the antibody response

For Western blots, sera of rats during acute and chronic infection were analysed; immunoglobulins A, G2b, G2a, and G1 were selected because of their relative dominance in the respective infection phases (Fig. 4). Using both sporozoite and bradyzoite-antigens, Western blots revealed that early IgA antibodies only reacted with sporozoites recognising a relatively broad range of molecules (Fig. 4a); antigens at 84, 75, 54 and 33 kDa were consistently observed. Early IgG2b antibodies reacted with both parasite stages. Runs with sporozoite-antigens showed a band at 24 kDa, two bands at 27 kDa, and a weak, but consistently visible antigen at 58 kDa (Fig. 4b). In electrophoretic runs using bradyzoite extracts, IgG2b detected a major band at 75 kDa and bands at 38 and 98 kDa (Fig. 4b). IgG2a antibodies collected during the acute phase reacted predominantly with a sporozoite antigen at 71 kDa whereas reactivity with bradyzoite antigen was weak (Fig. 4c). The reaction profiles of these isotypes and subclasses from sera of chronically (60 days p.i.) infected rats were similar except that IgG2b antibodies showed a stronger reaction towards bradyzoite antigens and parasite-specific IgA reacted with a bradyzoite antigen at 75 kDa (Fig. 4d).

According to the ELISA results, in particular of experiment 2, parasite-specific IgG1 constituted a considerable component of the B cell response during the chronic phase of infection. This was confirmed by the Western blots because strong reactions were seen with both sporozoite and bradyzoite antigens (Fig. 4e), in particular with a major sporozoite antigen at 79 kDa and several bands around 71 kDa in bradyzoites.

Because molecules around 70 kDa appeared to be a major target of the B cell response, we probed parallel runs of the antigen preparations for reactivity with total IgG and polyclonal antibodies prepared against DnaK, the bacterial homologue of hsp70. A distinct band was detected with anti-hsp70 antibodies at 79 kDa in sporozoite but not bradyzoite extracts, and this co-migrated with a similar molecule recognised by IgG in sporozoites (Fig. 4f). Therefore, it is possible that a part of the antibody response was directed against a parasite-hsp70, for instance in the case of IgG1 (Fig. 4e). The Western blot further showed that the 79 kDa molecule of sporozoites recognised by IgG migrated differently from bands detected in a similar molecular range of bradyzoite extracts indicating that these antigens were probably not identical in both stages.

Parasite-specific antibodies rarely showed reactivity with molecules around 30 kDa in either parasite stage (excep-
tion: IgG2b with sporozoite antigen), and no reaction was seen ≤20 kDa. This was due to an absence of reactivity, not incomplete transfer of proteins from gels to nitrocellulose which was confirmed by staining of nitrocellulose strips with Ponceau. The extracts of sporozoites as well as bradyzoites showed a variety of molecules in the low molecular weight range after electrophoretic separation (Fig. 5b).

Fig. 4. Western blot analysis of the antibody response of wild brown rats to *S. singaporensis*. Relative molecular weight is indicated as kDa. Analysis was performed for immunoglobulins IgA (a) (16 days p.i.), IgG2b (b), IgG2a (c) (both 24 days p.i.), the former three immunoglobulins at 60 days p.i. (d), and IgG1 (e) (60 days p.i.). Lanes 1–4 indicate electrophoretic runs with sporozoite extracts and lanes 5–8 with bradyzoite extracts, except in Fig. 4d where lanes 1 and 2 refer to sporozoite and lanes 3 and 4 to bradyzoite antigen. Each lane comprises a pool of two representative sera. Lanes 4 and 8 show negative controls, except in Fig. 4d where these are shown in lanes 2 and 4. (f) Detection of hsp70 using anti-DnaK antibodies and reactivity of total IgG (60 days p.i.) in parallel runs of sporozoite antigen (lanes 1–3) versus bradyzoite antigen (lanes 4–6). Lanes 1 and 4, anti-DnaK; lanes 2 and 5, IgG of infected rats; lanes 3 and 6, IgG of uninfected rats. Lanes show a pool of three representative sera. Anti-DnaK antibodies were probed in two independent experiments.
3.4. Antibody response to bradyzoite antigen of an avirulent and wild-type strain

The apparent lack of reactivity of sera in the low molecular weight range was intriguing. We therefore compared sera of rats infected with the wild-type strain, S5, with those infected with an avirulent strain of *S. singaporensis*, S1. As shown in Fig. 5a, infection of wild brown rats with the avirulent strain resulted in about 1/12 of the numbers of bradyzoites as found for the wild-type 8 weeks p.i. Such a reduction of bradyzoites, the stages which are eventually responsible for transmission of the infection to the definitive host, results in reduced sporocyst development in the definitive host and, sometimes, fails to induce a patent infection at all (T. Jäkel, unpublished observation). Profiles of extracts of bradyzoites digested free from the muscles of the infected rats showed distinct differences in protein expression between strains S1 and S5, in particular an apparent overexpression of molecules at 26 and 42 kDa in S1 (Fig. 5b). Western blots of the strain-specific IgG response to bradyzoites revealed that several antigens in the low molecular weight range were recognised in the avirulent strain, but not in the wild-type; this was observed in two independent experiments. A strong reaction with antigens of strain S1 occurred at 31, 20, 17, and 15 kDa (Fig. 5c). The reaction profiles of total IgG with wild-type bradyzoites confirmed the results obtained by the subclass-specific Western blots and indicated that also IgG2c antibodies (which were excluded from the previous analysis) did not react with antigens of low mol. wt. Considering the possibility that these molecules were not expressed in the wild-type parasites and, therefore, not immunogenic in rats, we probed rabbit-IgG obtained after subcutaneous immunisation of a New Zealand rabbit with wild-type bradyzoites. As shown in Fig. 5c, the rabbit antibodies reacted with molecules in the low molecular weight range of bradyzoites of both strains similar to those recognised by the rat antibodies directed against the avirulent strain. This indicated that these antigens were expressed in the wild-type parasites and, at least, immunogenic in a host not susceptible to infection with *S. singaporensis*.

4. Discussion

The present investigation provides evidence that acute infection with a *Sarcocystis* species induces a Th1-type antibody response in the intermediate host, namely elevated levels of parasite-specific IgG2b and IgG2c in rats (Gracie and Bradley, 1996). This is followed by a concomitant increase of IgG1 during chronic infection resulting in a merged Th1/Th2 pattern. In experiment 1, significant concentrations of parasite-specific IgG2a appeared at the end of the acute phase, and the presence of elevated concentrations of IgG2a in immunised rats indicates that this subclass could play a role during early infection already. These observations conform with the view that IgG2a is influenced positively by Th1 cytokines in rats (Uchikawa et al., 1994; Gracie and Bradley, 1996; Smith et al., 1997). The B cell response to acute *Sarcocystis* infection shows similarities to what has been observed for *Plasmodium berghei* in rats (Smith et al., 1997). However during acute infection, parasite-specific IgG2b concentrations generally appeared to be higher than those of the other IgG2 subsets. Our results also largely conform with observations on *Toxoplasma gondii* in rats where IgG2b is the predominant subclass (Zenner et al., 1999). Immunoglobulin G2b has
been identified as the most effective subclass for mediating complement-dependent lysis (Hughes-Jones et al., 1983) and binding to macrophages (Denham et al., 1987). A further similarity between \textit{Sarcocystis} and \textit{Toxoplasma} is the early concomitant increase of IgM and IgA antibodies after infection (Goddard et al., 1990) whereby the latter is readily explained with the peroral route of infection, and, in the case of \textit{S. singapourensis}, early immune reactions in the lungs (Jäkel et al., 1996). However, we have no plausible explanation for the considerable variation of the IgA response in our experiments, particularly during chronic infection, or, the presence of elevated concentrations in uninfected rats (Fig. 2). The latter may be attributed to cross-reactive properties of IgA antibodies directed against other infectious agents in the wild animals. IgG1 antibodies were not detected during chronic toxoplasmosis and were only elicited if rats were immunised with certain soluble parasite antigens (Zennor et al., 1999). Such a relation between soluble proteins and the IgG1 response is also known for other infection models (Coutelier et al., 1991). Therefore, one could speculate that IgG1 could be, at least in part, a response to soluble parasite antigens during chronic sarcocystosis. It has been shown for \textit{S. singapourensis} that sarcocysts rupture and are degraded as early as 8 weeks p.i. (Häfner, 1987), events that are likely to release soluble parasite components.

Although one could simply explain the observed antibody kinetics as composed of a reaction against schizogenic stages (IgG2) and a reaction against sarcocysts in the muscles (IgG2/IgG1), the situation is probably more complex. The Western blots show, for instance, that IgG2b antibodies (of the acute phase) recognised antigens in sporozoite as well as bradyzoite extracts, and the same was true for IgG1 collected during chronic sarcocystosis. These observations only make sense in the light of new discoveries concerning the life cycle of \textit{Sarcocystis}. Until recently, it was assumed that there is a straight developmental sequence from sporozoites via merozoites to bradyzoites (Dubey, 1993). We have extended this view with the discovery of the synchronous development of two different subpopulations of merozoites during acute infection in the rat, where one subpopulation predominantly expresses sporozoite antigens (type 1 merozoites) and the other bradyzoite-related antigens (type 2 merozoites) (Jäkel et al., 1999a). The latter could explain why we measured early parasite-specific antibody responses in rats despite employing bradyzoite-antigen in the ELISA, and, furthermore, the reactivity of IgG2b antibodies of the acute phase with bradyzoite-antigens in Western blots. On the other hand, IgG1 antibodies reacted with sporozoite-antigens at a time (60 days p.i.) when sporozoites and type-1-merozoites are not expected to be present in the host anymore. There are two possible explanations for this: first, sporozoites or type-1-merozoites could still be present in the chronic phase or, second, antigen of these stages is retained, e.g. in dendritic cells (Tew et al., 1980), for a long time after they have disappeared.

Using polyclonal antibodies prepared against DnaK, the bacterial homologue of hsp70, we identified a heat shock protein, or heat shock-related molecule, at 79 kDa in antigen extracts of sporozoites. It appeared that parasite-specific rat IgG recognized a similar molecule in sporozoites suggesting that a part of the antibody response could be directed against a heat shock protein. However, this must be further validated by screening of rat sera with a recombinant or otherwise purified heat shock protein of the parasite, which is not currently available. Heat shock proteins are among the dominant antigens recognized by the immune system for a large spectrum of parasites (Engman et al., 1990; Maresca and Carratu, 1992) and the identification of a similar molecule in \textit{Sarcocystis} would not come as a surprise.

An intriguing result of the present study is the observation that the B cell response of wild brown rats to \textit{S. singapourensis} rarely recognised molecules around 30 kDa (exception: IgG2b and sporozoite extracts), and nearly no reactivity occurred with antigens \(\approx 20\) kDa. If one considers that certain surface proteins of apicomplexan parasites at around 30 kDa are among the most prominent antigens recognised by the immune system (Granstrom et al., 1990; Bülow and Boothroyd, 1991; Sommer et al., 1992) and molecules at low mol. wt. include antigens of organelles important for host cell invasion (Eisenbacher et al., 1993; Howe and Sibley, 1999), it appears that the B cells in the present infection model were unresponsive to these antigens, which are likely to be expressed in \textit{S. singapourensis} as well (and actually were expressed, Fig. 5c). We show here for the IgG response to bradyzoites that the use of an avirulent strain instead of the wild-type for infection abrogates this unresponsiveness. The antibody response of wild rats to the avirulent strain of \textit{S. singapourensis} appeared to be stronger compared with the wild-type as additional antigens of low mol. wt. were detected. The reason for this is unclear, but one could speculate that the wild-type parasites employ an immune evasion strategy which would eventually support the parasite’s development.

Wild rats can mount a rapid specific immune response to lethal infection dosages of \textit{S. singapourensis}, if primarily infected a few days before challenge, inhibiting development of intracellular schizonic stages in the lungs within approximately 15–17 days (Jäkel et al., 1996). The results of the present challenge experiment conform with this, showing a rapid antibody response exhibiting an immunoglobulin subclass profile indicative of an underlying cellular immune response. In contrast, laboratory rat strains infected with pathogens establish immunity to infection in terms of several weeks, not days (Knopf et al., 1977; Marrack and Kappler, 1994; Zennor et al., 1999). Interestingly, the main difference between protected and non-protected wild brown rats was significantly elevated concentrations of parasite-specific IgG2a in the former. Although this does not necessarily indicate a protective role for IgG2a, it might point to an important target of the rat’s immune system. As revealed by the Western blots, IgG2a predominantly reacted with
sporozoite antigens, even during chronic infection. Early proliferating merozoites share most of their antigenic properties with sporozoites (Jäkel et al., 1999a) and are likely to be a major target of the immune response because their numbers in the lungs determine the pathological consequences of infection (Jäkel et al., 1996). With regard to the latter, it is interesting that lymphocytes isolated from pulmonary hilar lymph nodes showed the highest level of IgG2a production in another infection model (Uchikawa et al., 1994).

Further research into the T cell response to S. singaporiensis will be necessary to determine the relative roles of antibodies and cellular immunity in controlling schizogenic development. An important role has been assigned to CD8+ T cells for controlling infections with coccidians (Milon and Louis, 1993).

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