Genetic influence on the kinetics and associated pathology of the early stage (intestinal-hepatic) migration of *Ascaris suum* in mice

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SUMMARY

The generative mechanism(s) of aggregation and predisposition to *Ascaris lumbricoides* and *A. suum* infections in their host population are currently unknown and difficult to elucidate in humans and pigs for ethical/logistical reasons. A recently developed, optimized murine model based on 2 inbred strains, putatively susceptible (C57BL/6j) and resistant (CBA/Ca) to infection, was exploited to elucidate further the basis of the contrasting parasite burdens, most evident at the pulmonary stage. We explored the kinetics of early infection, focusing on the composite lobes of the liver and lung, over the first 8 days in an effort to achieve a more detailed understanding of the larval dispersal over time and the point at which worm burdens diverge. Larval recoveries showed a heterogenous distribution among the lobes of the lungs, being higher in the right lung of both strains, and in the susceptible strain larvae accumulating preferentially in 2 (caudal and middle) of the 4 lobes. Total larval burdens in these 2 lobes were largely responsible for the higher worm burdens in the susceptible strain. While total lung larval recoveries significantly differed between mouse strains, a difference in liver larval burdens was not observed. However, an earlier intense inflammatory response coupled with more rapid tissue repair in the hepatic lobes was observed in CBA/Ca mice, in contrast to C57BL/6j mice, and it is possible that these processes are responsible for restricting onward pulmonary larval migration in the resistant genotype.

Key words: *Ascaris suum*, mouse model, C57BL/6j, CBA/Ca, susceptibility, migration, liver, lungs, lobar distribution, inflammatory reaction.

INTRODUCTION

Ascaris lumbricoides and *Ascaris suum* are important widespread parasitic nematodes that cause infections of humans and pigs respectively (O' Lorcain and Holland, 2000; Crompton, 2001). *Ascaris* exhibits an aggregated frequency distribution in its hosts (Croll and Ghadirian, 1981; Boes *et al*. 1998; Holland and Boes, 2002). Furthermore, observations of re-infection patterns post-chemotherapy have indicated that individuals display a degree of predisposition to their worm burden status (Haswell-Elkins *et al*. 1987; Holland *et al*. 1989; Keymer and Pagel, 1990; Boes *et al*. 1998). The generative mechanism(s) of these observed phenomena are currently unknown and are difficult to elucidate in humans and pigs for ethical and logistical reasons. Recent studies have proposed that host genetics play a role in predisposition although precisely which genes are involved and what they control is still largely unknown. One possibility is that genes influencing immune mechanisms that control the intensity of infection play a key role (Holland *et al*. 1989, 1992; Williams-Blangero *et al*. 2008; Nejsum *et al*. 2009), and conceivably together with physiological factors, contribute to the processes that are responsible for predisposition (Mc Sharry *et al*. 1999; Holland and Boes, 2002). Inevitably these processes are likely to be confounded further by the influence of environmental factors such as nutrition and concurrent infections which are known to affect susceptibility to infection (Keymer and Pagel, 1990). Predisposition to *Ascaris* infection has important implications for transmission, morbidity and control, and it is therefore crucially important to identify the mechanistic basis of resistance/susceptibility (Anderson and May, 1985) and their roles in predisposition to infection.

Comparative studies on larval migration have demonstrated that the mouse is a suitable model for investigating the early phase of *A. suum* infection (Murrell *et al*. 1997; Slotved *et al*. 1998). Mitchell *et al*. (1976) reported that mouse strains vary in their susceptibility to *Ascaris* infection and therefore provide a convenient model for investigating the

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genetic basis of variation in migratory kinetics and immune responses to infection. A murine model was recently developed and optimized and 2 inbred strains were identified as putatively susceptible (C57BL/6j) and resistant (CBA/Ca) to infection (Lewis *et al.* 2006). This was reflected in the distinct, repeatable difference in *A. suum* larval burden in the lungs on day 7 post-infection (Lewis *et al.* 2006). The contrast of resistance and susceptibility between CBA and C57BL/6j mouse strains respectively represents the extremes of host phenotype displayed in an aggregated distribution.

The underlying mechanism responsible for the loss of *A. suum* larvae during primary infections and its precise location within the body are currently unknown. Recent studies undertaken by Lewis *et al.* (2006, 2007) revealed that a similar numbers of larvae were detected in the liver of both mouse strains. The majority of the larvae observed in the liver successfully migrated to the lungs in the C57BL/6j, while far fewer larvae accumulated in the lungs of CBA/Ca mice. Subsequent examination of the leukocyte population in bronchoalveolar lavage (BAL) fluid and lung histopathology indicated that whilst there were clear differences in the intensity of the response between strains, the pulmonary inflammatory reaction was unlikely to play a prominent role in explaining the difference between *Ascaris* larval burdens in these mouse strains (Lewis *et al.* 2007). The liver has previously been cited as a key site in the immobilization of migrating *Ascaris* larvae in mice (Sprent and Chen, 1949; Taffs, 1968; Mitchell *et al.* 1976; Johnstone *et al.* 1978; Song *et al.* 1985), guinea pigs (Kerr, 1938; Fallis, 1948) and rabbits (Arean and Crandall, 1962).

The white spot (WS) lesions that form as a result of mechanical injury and inflammatory response induced by migrating *Ascaris* larvae in the liver have been suggested to form along the larval migration routes or encapsulate trapped larvae (Ronéus, 1966; Copeman and Gaafar, 1972; Peréz *et al.* 2001). Therefore WS have been proposed to play a role in immunity to *A. suum* infection in pigs (Copeman and Gaafar, 1972; Eriksen *et al.* 1980). *A. suum* larval debris has been detected within WS (Schwartz and Alicata, 1932; Sprent and Chen, 1949; Copeman and Gaafar, 1972; Peréz *et al.* 2001).

There has been little focus on the larval distribution within the various lobes of the liver and lungs. McCraw and Greenway (1970) recovered *A. suum* larvae from lung lobes in calves but stated that no significant difference between the pulmonary lobes was detected. The larval and adult intestinal burden has been quantified in pigs (Eriksen *et al.* 1992; Murrell *et al.* 1997; Roepstorff *et al.* 1997; Jungersen *et al.* 1999) and in model organisms such as mice and guinea pigs (Jenkins, 1968; Keittivuti, 1974). Furthermore, larvae of *A. suum* have been observed in the wall and lumen of large intestinal segments, the caecum, colon and rectum in mice (Jenkins, 1968; Douvres and Tromba, 1971; Keittivuti, 1974; Lewis *et al.* 2007) and pigs (Murrell *et al.* 1997).

The main aim of the present study was to determine if the liver plays a significant role in parasite attrition in resistant mice. In order to assess the precise times and locations of larval attrition and accumulation, during the tissue-migratory phase of *Ascaris* infection in C57BL/6j and CBA/Ca mice, the distribution of larval numbers was quantified in the small and large intestines and in individual hepatic and pulmonary lobes. Moreover, the role of hepatic inflammation in explaining variation in susceptibility between the two inbred mouse strains was determined by semi-quantitative scoring of the histopathological changes within the liver.

MATERIALS AND METHODS

Experimental animals

A total of 180 (90 C57BL/6j and 90 CBA/Ca) male inbred mice was purchased from Harlan UK Ltd at 7 weeks of age. They were allowed a week to acclimatize to animal house conditions before infection at 8 weeks of age. Mice were housed in an animal maintenance room in the Bioresources unit, Trinity College Dublin for the duration of the experimental procedure. The room was maintained at approximately 22 °C with a daily 12 h light/dark photoperiodicity. Water and commercial pelleted food were supplied *ad libitum*, and cages were cleaned on a regular basis. The mice were individually weighed on arrival, and randomly assigned, within each strain, to groups of 4 per cage.

Parasite

Approximately 5 000 000 embryonated *Ascaris suum* ova (batch no AP 04.04) were supplied by the Danish Centre for Experimental Parasitology (CEP), Copenhagen. Individual doses were adjusted in order to contain 1000 fully embryonated ova in 0·1 M H_2SO_4 as described previously (Lewis *et al.* 2006). Placebo doses for control mice consisted of 0·9% saline only.

Infection and post-mortem of mice

Upon commencement of infections, mice were randomly assigned as experimental (infected: 72 C57BL/6j and 72 CBA/Ca) and control (not infected: 18 C57BL/6j and 18 CBA/Ca) mice. In order to facilitate larval counts from the intestines, liver and lungs of both mouse strains, 45 experimental mice of each strain were designated for this purpose, while the remainder of the experimental mice (27 of each strain) and all control uninfected mice were assigned for histopathological examination of the

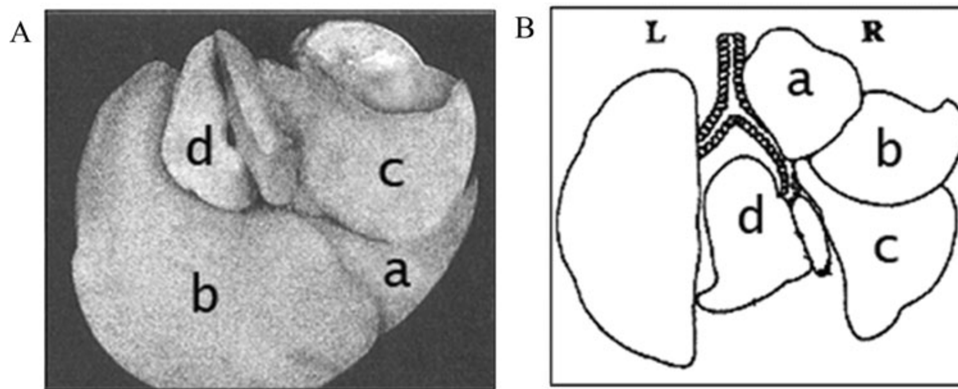

Fig. 1. The composite lobes of the (A) murine liver (a, median lobe; b, left lobe; c, right lobe; d, caudate lobe) and (B) murine left (L) and right (R) lungs (a, cranial lobe; b, middle lobe; c, caudal lobe; d, accessory lobe) (adapted from Maronpot *et al.* 1999).
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liver. Infective *A. suum* ova and placebo doses were administered by gastric intubation to experimental and control mice respectively. Post-mortems were conducted at 6 h and on days 1–8 post-infection (p.i.). At each post-mortem time-point 8 experimental mice (5 for larval counts and 3 for histopathological examination) and 2 control mice of each strain were euthanized by cervical dislocation. Prior to euthanasia, mice were individually weighed.

Larval counts

Five mice of each strain were euthanized at each post-mortem time-point and viable larvae were enumerated in the large and small intestines, liver and lungs. The small intestine was separated into 7 cm segments (1–7 cm, 8–14 cm, 15–21 cm and the remainder, 22-x cm) from the pylorus of the stomach. The large intestine was also divided into the caecum and colon. The liver was separated into its multiple lobes, the median, caudate, left and right lobes. Similarly, the right lung was separated into its composite lobes, the cranial, middle, caudal and accessory lobes. The left lung cannot be further separated (Fig. 1). All lobes and intestinal segments were evaluated as separate entities.

Living larvae were recovered from each lobe and segment by means of the modified Baermann technique (Lewis *et al.* 2006). A pellet of the isolated viable larvae was suspended in a 5 ml solution of 0·9% saline and 6% formalin. Prior to larval counts the 5 ml solutions were agitated to ensure a homogenous distribution of larvae within the sample. Larval counts were conducted on 1 ml of each sample. In order to enumerate the live larvae in the liver, large and small intestinal samples, twenty 50 μl, ten 100 μl and five 200 μl aliquots respectively were screened under ×40 magnification. When recording the larval burden in the lung samples, 2 ml were pipetted into the chamber of a nematode counting slide (Chalex Corporation). The number of larvae present in the grid area, which represents 1 ml, was counted under ×40 magnification. The number of larvae in a 1 ml solution was multiplied by total volume in order to estimate the number of larvae in the tissue sample.

Histopathological examination

Three experimental and 2 control mice of each strain were euthanized at each post-mortem time-point. The liver was separated into individual lobes as outlined above, fixed in 10% buffered formalin for 24 h. Following paraffin embedding, 5 μm sections of each lobe were cut, stained with haematoxylin and eosin, and examined. Hepatic injury and inflammation was scored semi-quantitatively ranging from +/− (no inflammation) and + (very mild inflammation) to ++++ (severe inflammation). Examining sections of each hepatic lobe from each animal reduced the margin of error in scoring that could have resulted from a localized response. Histopathological examination was performed without prior knowledge of sample identity.

To observe changes in the hepatic inflammatory reaction an initial histopathological section from each hepatic lobe from each animal sampled at 6 h and days 1–8 p.i. was examined and graded. A further 3 histopathological sections were taken at a fixed distance of 100 μm apart from each lobe from each animal euthanised on days 2–5 p.i. The scores allotted to the 3 further serial histopathological sections examined were subjected to statistical analysis.

Statistical analysis

All statistical analysis was carried out at the 95% confidence limit. Larval recovery data were assessed for normality and were subsequently log transformed. Intestinal larval numbers were not subjected

Table 1. Mean larval numbers (±S.E.M) recovered at 6 h and 1–8 days p.i. in the large and small intestines in C57BL/6j and CBA/Ca mice, following inoculation with 1000 *Ascaris suum* ova

Day p.i.	Large intestine						Small intestine									
	Caecum		Colon		Total		1–7 cm		8–14 cm		15–21 cm		22-x cm		Total	
	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca	C57BL/6j	CBA/Ca
6 h	102±24·0	180±45·3	7±4·4	8±4·9	109±24·4	188±45·3	0	2±2·0	0	0	0	1±1·0	2±1·2	0	3±1·2	3±2·0
1	1±1·0	0	0	2±1·2	1±1·0	2±1·2	0	0	0	1±1·0	0	0	0	0	0	1±1·0
2	1±1·0	0	3±1·2	1±1·0	4±1·9	1±1·0	0	0	0	0	0	0	0	0	0	0
3	0	0	1±1·0	1±1·0	1±1·0	1±1·0	0	0	1±1·0	0	0	0	0	0	1±1·0	0
4	0	0	0	2±1·2	0	2±1·2	0	0	0	0	0	0	0	0	0	0
5	0	2±1·2	1±1·0	1±1·0	1±1·0	3±1·2	1±1·0	0	0	0	0	0	2±1·2	0	3±2·0	0
6	2±1·2	0	1±1·0	3±2·0	3±1·2	3±2·0	2±1·2	0	1±1·0	0	0	0	1±1·0	0	4±1·9	0
7	0	0	0	0	0	0	0	1±1·0	0	0	0	0	0	6±4·9	0	7±5·8
8	4±2·9	1±1·0	2±1·2	0	6±2·9	1±1·0	0	0	1±1·0	0	1±1·0	1±1·0	1±1·0	4±2·9	3±3·0	5±2·7

to statistical analysis as larvae were almost exclusively recovered on a single post-mortem time-point. The influence of the factors, time and strain on the mean larval recoveries from the liver and lungs were investigated using a 2-way GLM (SPSS 15.0). To investigate the influence of the factors, time, strain and the distribution of larvae within organs on the mean larval recoveries in the lobes of the liver and the lungs, a MANOVA (SPSS 15.0) was conducted. In order to gauge whether a larval predilection for a specific lobe in an organ exists, a MANOVA was undertaken on the proportion of total larvae in each mouse recovered from each hepatic and pulmonary lobe. Proportional data were arcsine, square root transformed prior to analysis. Analysis of the semi-quantitative pathological scores (converted into a scale ranging from zero (+/−), to 4 (++++) in the liver was carried out using a mixed-GLM model, with time and strain as fixed factors, mouse as a random factor nested within the 2-way interaction of time∗strain and liver lobe as a fixed effect. The model was therefore time+strain+time∗strain+mouse (time∗strain)+liver lobe, with mouse as a random factor. We also explored models involving interactions between liver lobe and time and strain, but none of these proved significant. Appropriate statistics are reported in the text.

RESULTS

Ascaris suum migration in the intestines, liver and lungs of C57BL/6j and CBA/Ca mice at 6 h and 1 to 8 days p.i.

Larvae were detected at 6 h and on days 1–8 p.i. in the intestines, liver and lungs of both mouse strains, but larval burdens varied over time and differed between mouse strains.

The highest presence of larvae in the intestines of both mouse strains was recorded at 6 h p.i. At this time-point, the majority of larvae was recovered from the large intestine and had a strong predilection for the caecum as can be seen in Table 1. Mean larval burdens were higher in the resistant CBA mice. The mean larval numbers in the large intestine then decreased by day 1 p.i. in both strains and no consistent pattern distinguishing the two strains was evident (Table 1).

Ascaris suum larvae were recovered from the liver at every post-mortem time-point in each mouse strain. Appreciable numbers were recovered in this organ at 6 h and marked decreases were observed on days 4 and 5 p.i. in C57BL/6j and CBA/Ca mice respectively. A 2-way GLM demonstrated that the total numbers of larvae observed in the liver did not differ significantly between the two strains (main effect of strain, $F_{1,71} = 3{\cdot}060$, $P = 0{\cdot}085$). However, recoveries of larvae differed significantly over post-mortem time-points (main effect of time, $F_{8,71} = 23{\cdot}785$,

Table 2. Mean larval numbers (±S.E.M) recovered at 6 h and 1–8 days p.i. in the liver of C57BL/6j and CBA/Ca mice, following inoculation with 1000 *Ascaris suum* ova

Day p.i.	C57BL/6j	CBA/Ca
6 h	32±5·1	63±14·2
1	98±15·6	88±18·5
2	161±21·9	67±11·9
3	97·5±24·4	70±18·8
4	98±20·0	57±10·7
5	51±17·8	89±16·3
6	48±8·7	31±7·0
7	11±2·9	3±2·0
8	14±5·3	17±3·7

$P<0{\cdot}001$). A significant strain*time interaction was found ($F_{8,71}=2{\cdot}745$, $P=0{\cdot}011$) as the larvae recovered from each strain displayed different migratory patterns through this organ (Table 2).

Larvae were recovered in each liver lobe as early as 6 h p.i. In C57BL/6j the mean number of larvae peaked on days 1–3 p.i. but was dependent on the hepatic lobe and subsequently declined to comparatively low larval numbers on days 7 and 8 p.i. (Fig. 2). As in C57BL/6j mice, low larval numbers were observed on the later days of infection in the CBA/Ca liver lobes (Fig. 2).

The results from a 2-way MANOVA on the proportion of total larvae recovered in the 4 hepatic lobes as opposed to the absolute mean numbers revealed that a significant strain effect was detected in the median ($F_{1,71}=5{\cdot}519$, $P=0{\cdot}022$) and right ($F_{1,71}=4{\cdot}238$, $P=0{\cdot}043$) lobes. The proportion of total liver larvae recovered in the median lobe of C57BL/6j mice increased to a peak on day 2 p.i. (0·26±0·07) and then fluctuated until days 7 and 8 p.i. at which point no larvae were recovered in this lobe. In CBA/Ca mice the proportion recovered in the median lobe fluctuated consistently with the lowest proportion recovered on day 4 p.i. (0·04±0·03) and the highest on day 3 p.i. (0·42±0·10). With the exception of days 2 and 4 p.i. a higher proportion of larvae was recorded in the median lobe of CBA/Ca mice. In the right lobe, the proportion of total liver larvae observed in C57BL/6j mice fluctuated consistently, peaking on day 8 p.i. (0·61±0·42). However in CBA/Ca mice the proportion of total larvae in the liver recovered in the right lobe increased to a peak on day 4 p.i. (0·46±0·10) and then decreased steadily.

The strain effect was approaching significance in the caudate lobe ($F_{1,71}=3{\cdot}732$, $P=0{\cdot}057$), in which the proportion of total liver larvae was seen to fluctuate slightly in both mouse strains. However, a noteworthy decrease in the proportion of larvae recovered in the caudate lobe in CBA/Ca mice was observed on day 7 p.i. (0·00) followed by a considerable increase on day 8 p.i. (0·43±0·17). The proportion of total liver larvae recovered in the right and caudate lobe significantly changed over time (right lobe: $F_{8,71}=2{\cdot}950$, $P=0{\cdot}007$, caudate lobe: $F_{8,71}=2{\cdot}437$, $P=0{\cdot}022$). A significant time*strain interaction was observed in the median ($F_{8,71}=2{\cdot}091$, $P=0{\cdot}048$) and right ($F_{8,71}=3{\cdot}085$, $P=0{\cdot}005$) lobes.

The larval migratory kinetics observed in the liver and lungs of C57BL/6j and CBA/Ca indicated a reduction of larvae in the liver coinciding with the arrival of larvae in the lungs on days 4 and 5 p.i. In C57BL/6j mice, larvae accumulated in the lungs to a peak on day 7 p.i. (125±21·7) and subsequently fell by day 8 p.i. (50±21·5). Larvae consistently increased to a maximum on day 8 p.i. (31±7·5) in CBA/Ca mice. A 2-way GLM conducted on larval numbers in the lungs on days 5 to 8 p.i. indicated a highly significant effect of strain ($F_{1,32}=16{\cdot}164$, $P<0{\cdot}001$) and time ($F_{3,32}=9{\cdot}025$, $P<0{\cdot}001$). No strain*time interaction was detected ($F_{3,32}=2{\cdot}507$, $P=0{\cdot}077$) as the pattern of larval migration in this organ was relatively similar in both strains.

Appreciable numbers of larvae entered the left and right lungs on day 5 p.i. In C57BL/6j mice larvae in each lung increased to a peak on day 7 p.i. and subsequently declined on day 8 p.i. Larvae recovered in the left lung of CBA/Ca mice increased to a maximum on day 8 p.i. A similar burden was recovered on days 7 and 8 p.i. in the right lung of CBA/CA mice (day 7 p.i.: 23±4·9, day 8 p.i.: 22±6·0) (Fig. 3).

A 2-way MANOVA conducted on proportional larval burdens on days 5–8 p.i. in the left and right lungs indicated that a strain effect was not detected in either the left ($F_{1,32}=0{\cdot}198$, $P=0{\cdot}659$) or right ($F_{1,32}=2{\cdot}124$, $P=0{\cdot}155$) lungs. The proportion of larvae in the lungs, which were recovered in the left and right lungs varied over time (left lung: $F_{3,32}=4{\cdot}193$, $P=0{\cdot}013$, right lung: $F_{3,32}=5{\cdot}994$, $P=0{\cdot}002$). On some post-mortem time-points, the proportion of larvae recovered in the right lung differed significantly between C57BL/6j and CBA/Ca mice ($F_{3,32}=5{\cdot}060$, $P=0{\cdot}006$).

The mean larval burden recovered on days 5–8 p.i. in the right lung lobes is illustrated in Fig. 4. In C57BL/6j larvae increased to a peak on day 7 p.i. in each lobe, which was also observed in the middle lobe in CBA/Ca mice. However, in the cranial, caudal and accessory lobe of CBA/Ca mice, larvae increased to a maximum on day 8 p.i.

A 2-way MANOVA conducted on the proportional data confirmed the effects of strain ($F_{4,29}=3{\cdot}576$, $P=0{\cdot}014$) and time ($F_{12,77}=2{\cdot}565$, $P=0{\cdot}007$) on lobar distribution in the right lung. A strain*time interaction was observed ($F_{12,77}=3{\cdot}285$, $P=0{\cdot}001$) as the percentage of larvae in the right lung recovered in the 4 lobes varied between strains at some post-mortem time-points. From the 4 right lung lobes, only the larval burden of the middle lobe changed over time ($F_{3,32}=8{\cdot}546$, $P<0{\cdot}001$). The migratory pattern of larval burdens differed significantly

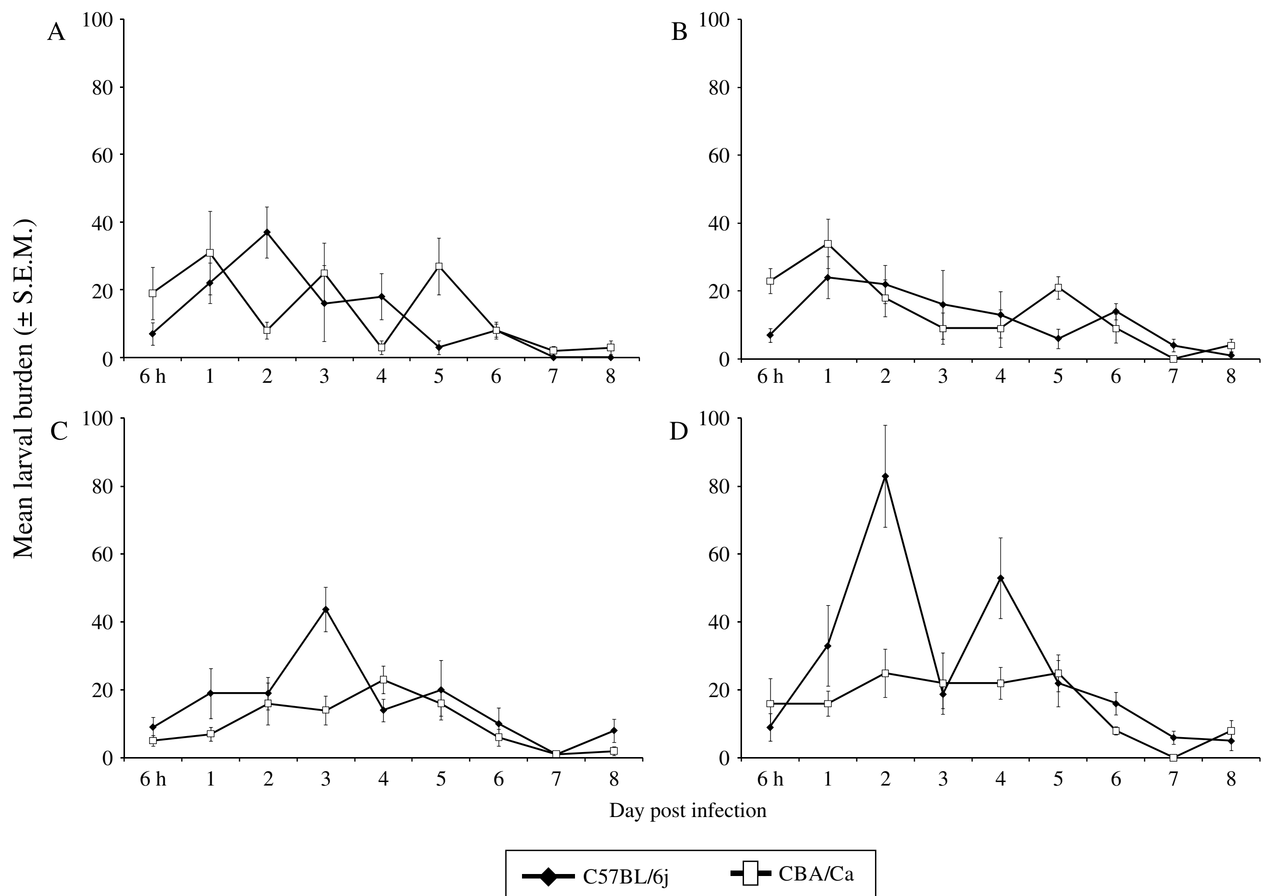

Fig. 2. Changes in total mean larval burden in the hepatic (A) median, (B) left, (C) right and (D) caudate lobes of C57BL/6j and CBA/Ca, following inoculation with 1000 *Ascaris suum* ova.

between the two mouse strains in the cranial ($F_{3,32}=3{\cdot}046$, $P=0{\cdot}043$) and middle ($F_{3,32}=3{\cdot}410$, $P=0{\cdot}029$) lobes. The proportional larval burden recovered in the caudal lobe was highly significantly different between C57BL/6j and CBA/Ca mice ($F_{1,32}=15{\cdot}331$, $P<0{\cdot}001$). The proportion of larvae in this lobe was consistently higher in C57BL/6j and the main sites for recovery in this mouse strain were the middle and caudal lobe. The disparity was most pronounced on day 6 p.i., when larvae were absent from the caudal lobe of CBA/Ca mice, and day 7 p.i., at which point 42·5% and 17·4% of the larvae were recovered from the caudal lobes of C57BL/6j and CBA/Ca mice respectively.

Histopathological examination of the liver

None of the uninfected control animals showed histopathological changes in the liver (Fig. 5A). Focal accumulations of granulocytes and macrophages were observed in infected animals with a predominant peri-portal and centrilobular distribution in each lobe but were also detected within the intervening hepatic lobular parenchyma (Fig. 5I). Necrotic tracts were widely distributed throughout the tissue with a particular association with portal and perilobular blood vessels (Fig. 5F). Longitudinal and transverse sections of *A. suum* larvae were frequently identified within blood vessels and sinusoids (Fig. 5F–H).

Semi-quantitative grading of sections from liver lobes (Fig. 5B–E) revealed a contrast in timing of the inflammatory reaction in C57BL/6j and CBA/Ca mice (Fig. 6A). A low inflammatory reaction score was recorded in both mouse strains at the initial stages of infection but was relatively greater in the CBA/Ca mice (mixed model as explained in the Materials and Methods section, main effect of strain $F_{1,16}=5{\cdot}0$, $P=0{\cdot}04$). Although C57BL/6j mice had a higher percentage of lobes with mild inflammation (grade +) than CBA/Ca mice, the C57BL/6j animals had extensive necrotic tracts radiating from blood vessels. Comparatively severe inflammation was observed in CBA/Ca mice on day 2 p.i., which became more pronounced on day 3 p.i. On day 4 p.i., more severe inflammation (grade ++++) was observed in more hepatic lobes of CBA/Ca animals. A similar response in the hepatic lobes in C57BL/6j mice was not detected until day 6 p.i. Comparatively less severe inflammation was detected on days 7 and 8 p.i. in CBA/Ca mice (Fig. 6A).

The mean semi-quantitative scores allotted to the 3 serial histopathological sections of each hepatic lobe illustrated a similar response in both mouse strains. In C57BL/6j mice the inflammatory reaction

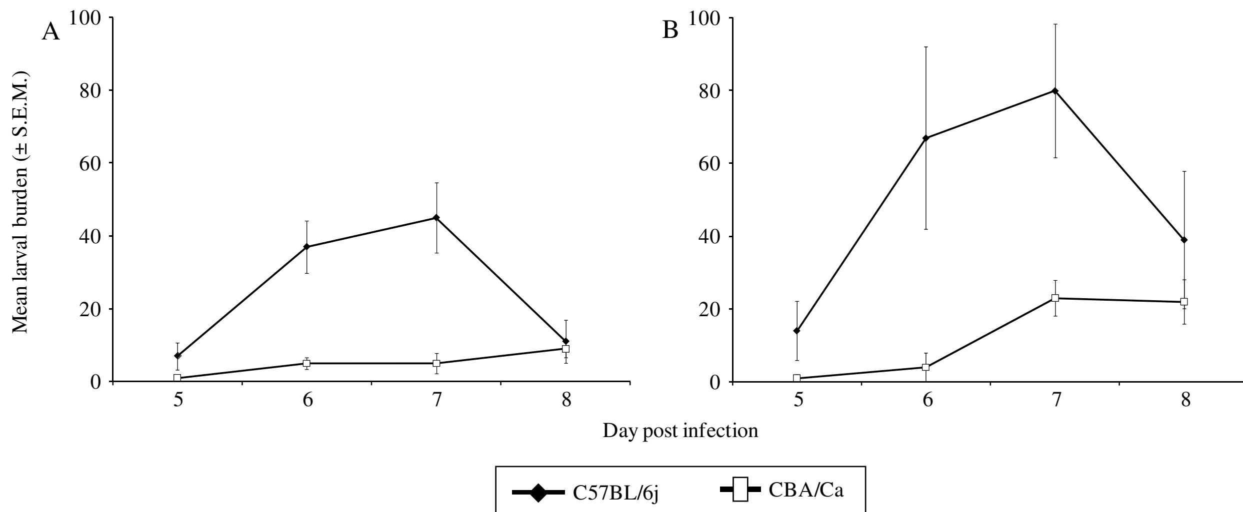

Fig. 3. Changes in total mean larval burden in the left (A) and right (B) lungs of C57BL/6j and CBA/Ca mice, following inoculation with 1000 *Ascaris suum* ova.

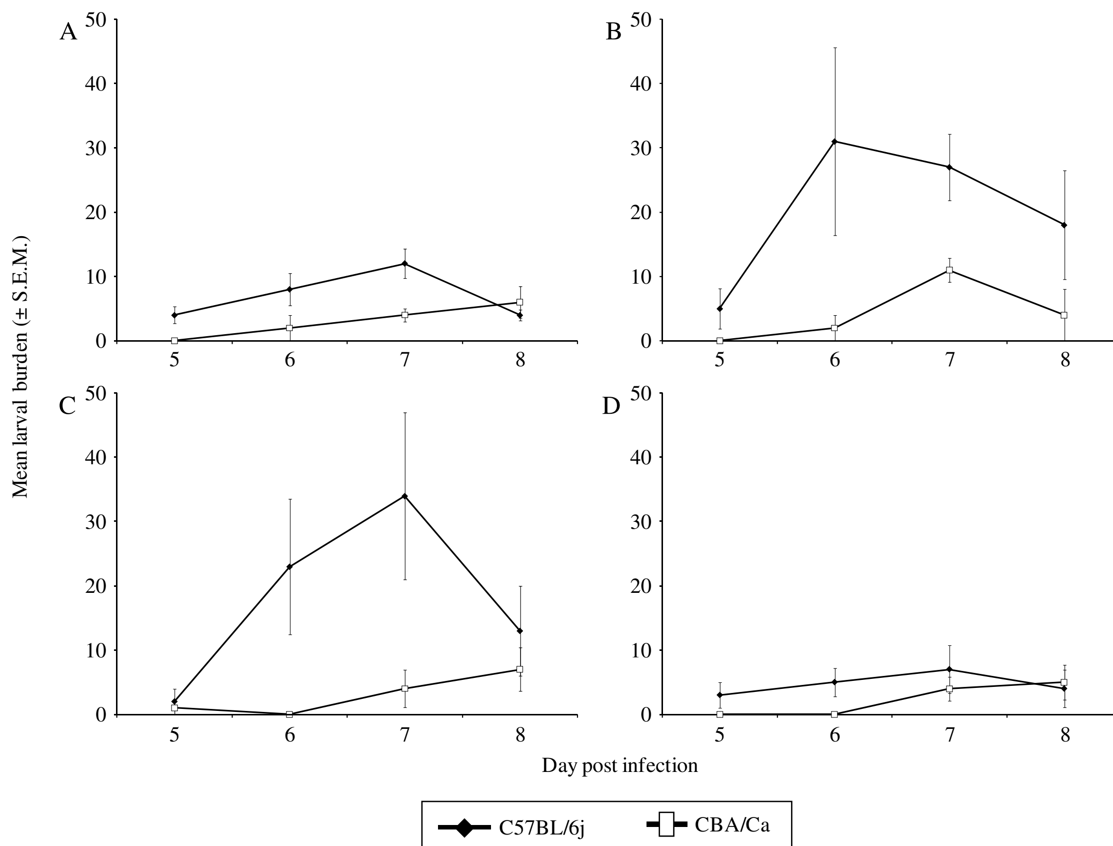

Fig. 4. Changes in total mean larval burden in the right lung (A) cranial, (B) middle, (C) caudal and (D) accessory lobes of C57BL/6j and CBA/Ca mice, following inoculation with 1000 *Ascaris suum* ova.

increased steadily over days 2–5 p.i in the left, right and caudate lobes. A similar inflammatory reaction was observed in the median lobe on days 3–5 p.i. In CBA/Ca mice the strong inflammatory reaction on day 4 p.i. and subsequent recovery on day 5 p.i. was observed in each hepatic lobe (Fig 6B).

These changes over time were significant (mixed model, main effect of time $F_{3,16}=8\cdot8$, $P=0\cdot001$), and both strains showed a similar pattern of changes, if not intensity, over time (2-way interaction time*strain, $F_{3,16}=2$, $P=\text{NS}$). There was no significant difference between liver lobes (main effect of liver lobe $F_{3,257}=1\cdot9$, $P=\text{NS}$) but with time, strain, the interaction between time and strain, and the main effect of liver lobe taken into account, there was a highly significant component of variation attributed to between-mouse variation in scores (mouse (time*strain), $F_{16,257}=10\cdot4$, $P<0\cdot001$).

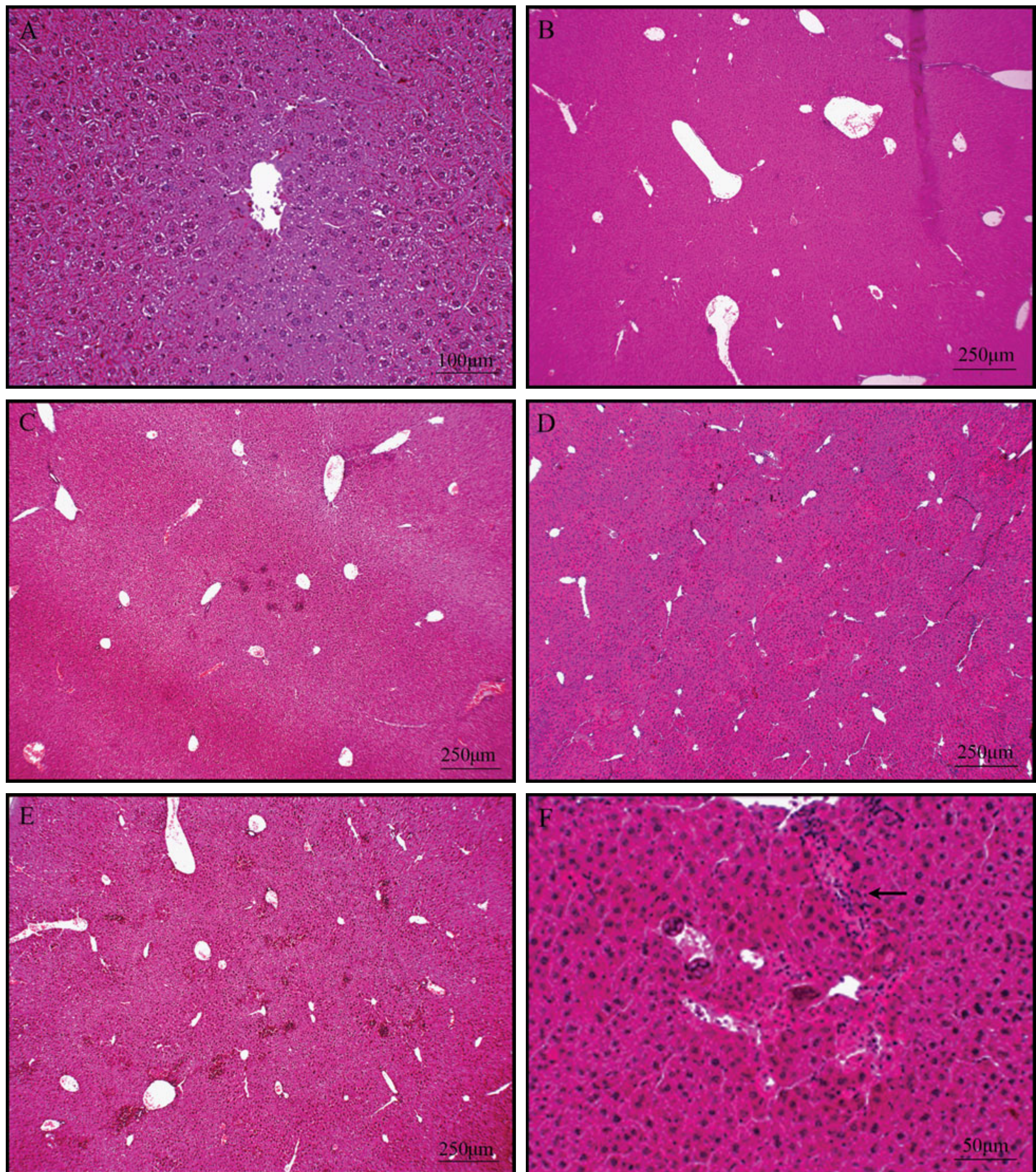

Fig. 5. (Cont.)

DISCUSSION

The results of the current study confirm and markedly extend earlier work indicating that migration of *A. suum* larvae is impeded in the liver or in between the liver and lungs in CBA/Ca mice. During migration in this study larvae were found to preferentially accumulate in particular lobes of critical organs in susceptible mice, thereby contributing to the difference in total larval burdens between strains of mice. Analysis of larval burdens in the organs through which the parasite migrates, particularly the significant disparity in lung larval numbers between strains and histopathological examination of the liver revealed a significant differential response, adding weight to the idea that the liver of resistant CBA/Ca mice plays an important role in the impediment of onward larval migration.

The importance of the large intestine in the early stages of the tissue migratory phase in *A. suum* infection in mice has been highlighted previously (Keittivuti, 1974; Slotved *et al.* 1998; Lewis *et al.*

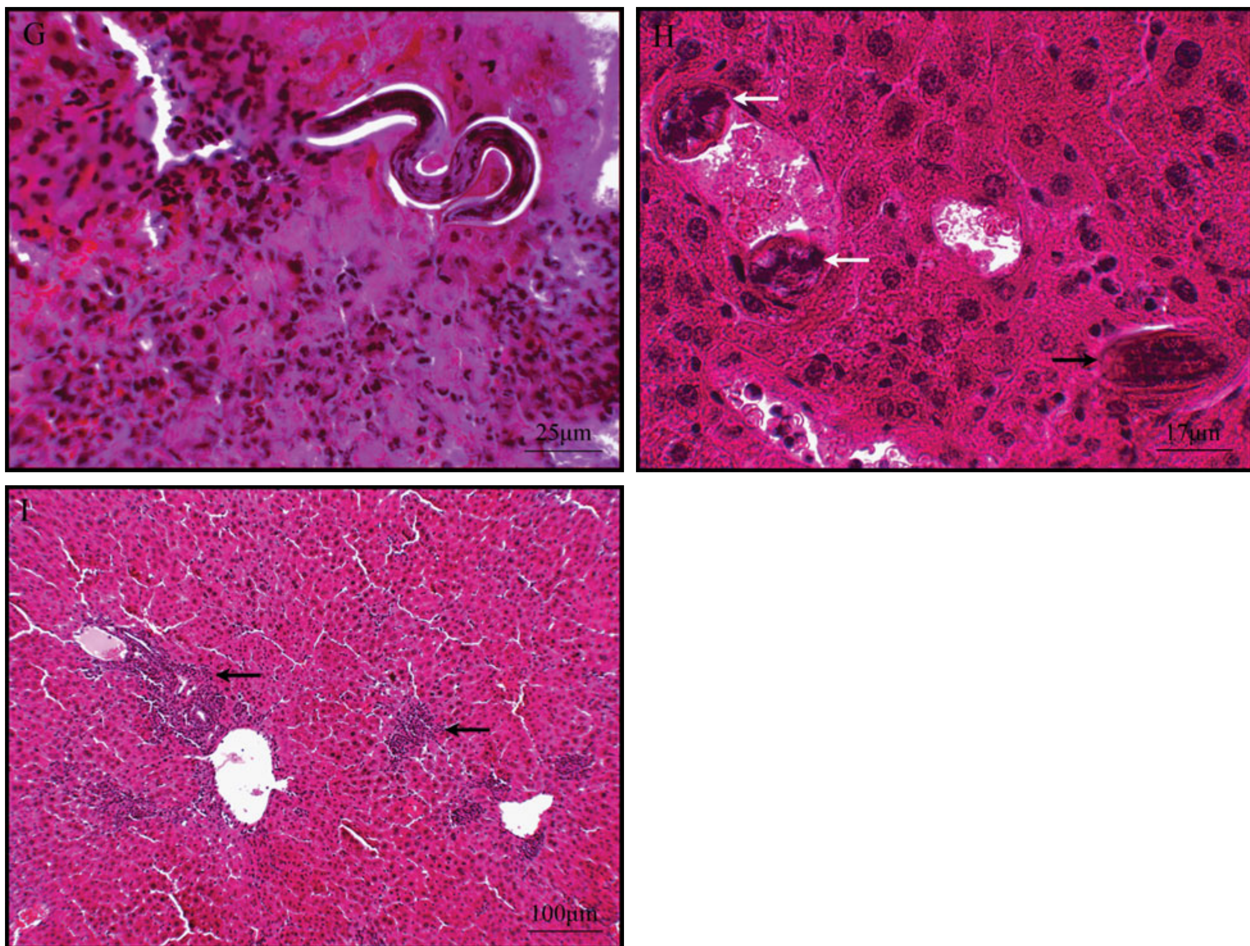

Fig. 5. Photomicrographs of haematoxylin and eosin-stained liver sections. (A) Control uninfected liver lobe (grade +/−), (B) mild inflammatory reaction (grade +), (C) moderate inflammatory reaction (grade ++), (D) strong inflammatory reaction (grade +++), (E) severe inflammatory reaction (++++), (F) Larvae with associated necrotic larval tracts (indicated by black arrow), (G) Longitudinal larva in hepatic parenchyma and nearby granulocytes, (H) Transverse (within blood vessel) (indicated by white arrows) and longitudinal (indicated by black arrow) (parenchymal) sections of larvae, (I) Focal accumulations of granulocytes in hepatic tissue (indicated by black arrows).

2007). In the current study, the number of larvae in the intestines was monitored over an 8-day period in susceptible and resistant mouse strains yet a high number of larvae was only recovered in the intestines at the 6-h p.i. time-point and was predominantly in the large intestine. The observed burden in C57BL/6j mice at this time in the large intestines was comparable to that of a previous study (Lewis *et al*. 2007). However, the larval burden detected in the large intestine of CBA/Ca mice was comparatively higher than in the C57BL/6j mice and also relative to the previously recorded larval burden in the large intestine of this strain at 6 h p.i. (Lewis *et al*. 2007). The observed high caecal numbers at the 6-h p.i. time-point was documented in mice by Keittivuti (1974) and Jenkins (1968), and in both of these studies a predilection for the caecal wall was noted.

Despite higher numbers in the large intestine of CBA/Ca mice at 6 h p.i., equivalent numbers in the liver suggest that a similar number of larvae successfully migrate from the large intestine to the liver in both mouse strains. This finding is consistent with earlier studies on our mouse model (Lewis *et al*. 2007) and in immunized and control guinea pigs (Soulsby, 1957). Timing, therefore, may account for the strain difference in the early intestinal burden as suggested by Slotved *et al*. (1998). These authors noted that individual mice of the same strain show variation in the rate at which larvae passed through the large intestine. While large intestinal *A. suum* larval burdens have been shown to peak at 6 h p.i. in mice (Keittivuti, 1974), larvae have been recorded in this tissue as early as 2 h p.i. (Keittivuti, 1974; Slotved *et al*. 1998). Furthermore, in the current study, larvae were recovered at 6 h p.i. in liver, indicating earlier hatching in the intestines. A more comprehensive examination of larval numbers in the large intestine at hourly intervals p.i. would indicate whether there is a temporal difference in hatching and larval penetration of the large intestinal wall. Nevertheless, if a temporal difference in hatching exists between the two strains, it is confined to a short period of time. This is reflected in the inconsistency displayed in the intestinal larval burdens on days

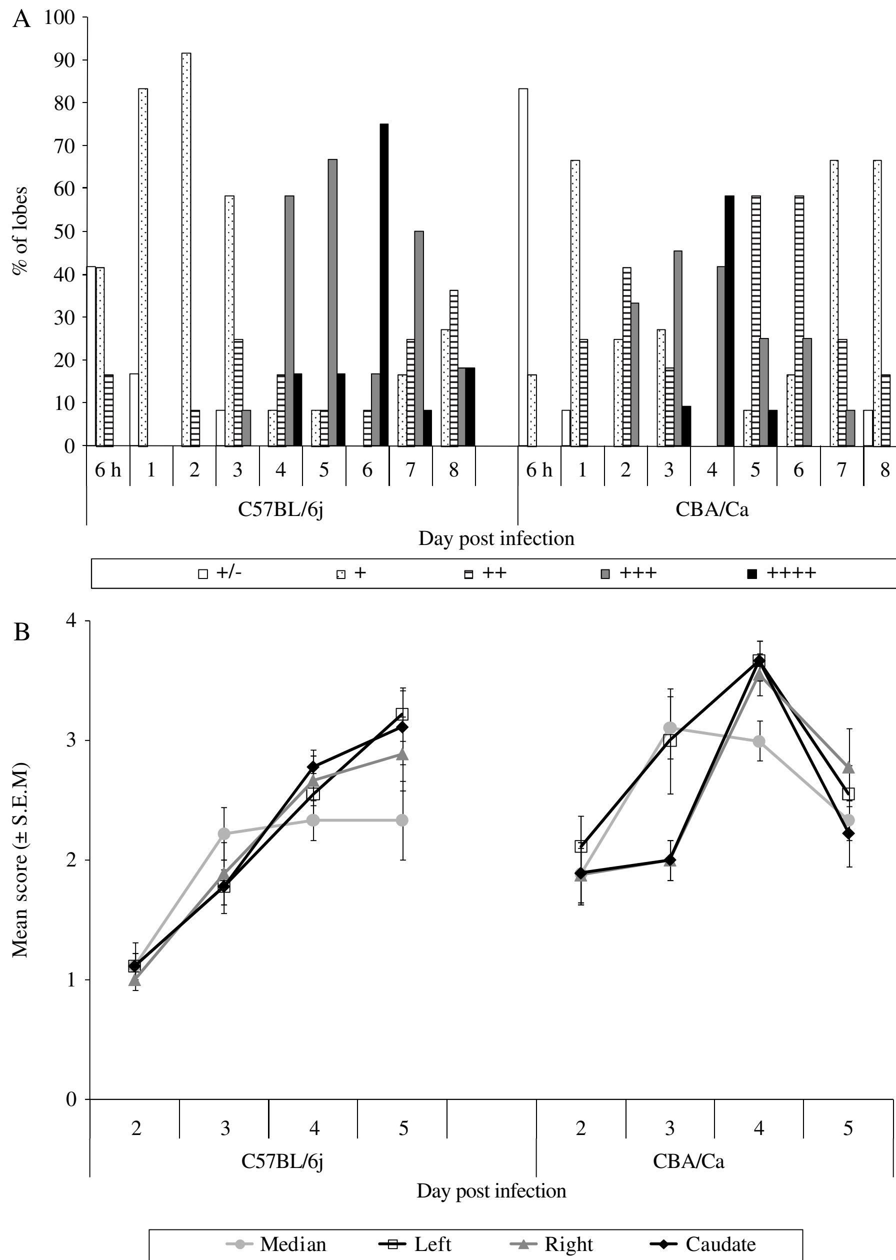

Fig. 6. Histopathological semi-quantitative scoring of inflammation in the hepatic lobes. (A) Changes in the percentage of hepatic lobes allotted each score based on examination of a single tissue section (B) Changes in the mean score allotted to each hepatic lobe based on examination of 3 serial tissue sections.

1–8 p.i. observed in our mouse model and up to 10 days p.i. in a previous study where the strain of mouse used was not specified (Keittivuti, 1974).

Ascaris suum larvae have been speculated to migrate within the liver of their host, for a period of days, in an attempt to find a suitable blood vessel for onward migration to the lungs. This was reflected in the erratic pattern of larval burden in each hepatic lobe, particularly the right and caudate lobes, in both strains. The pattern of migration within the liver was shown to differ between the two mouse strains in the median and right lobes. Furthermore, a difference in the proportion of total liver larvae was observed in the median and right lobes and was shown to be approaching significance in the caudate lobe. In an anatomical study of the mouse's liver, the caudate lobe was found to be the smallest, accounting for $8{\cdot}1 \pm 1{\cdot}0\%$ of the total liver in Balb/c mice, while the left lobe was shown to be the largest ($34{\cdot}4 \pm 2{\cdot}0\%$) (Inderbitzin *et al.* 2006). However, while no anatomical studies have been undertaken on the lobes of C57BL/6j and CBA/Ca mice, the large size of the left lobe does not explain the lack of disparity in the larval burdens in this lobe between the two mouse strains. The higher vascular density of the caudate lobe, observed in Balb/c mice (Inderbitzin *et al.* 2006) may vary between the two mouse strains exploited in this study, accounting for the increased larval accumulation in C57BL/6j mice. However, since Balb/c mice were found to be in the intermediate susceptibility

category in previous *A. suum* experiments (Lewis *et al.* 2006), this explanation seems unlikely.

It is currently not possible to determine if the difference in larval recovery from the hepatic caudate lobe is due to anatomical differences between the two mouse strains. Furthermore, the increased successful migration of *A. suum* larvae in C57BL/6j mice from the liver to the lungs may be explained by a hepatic or post-hepatic anatomical difference between the two strains. Strain-specific imaging of the liver and lungs may indicate barriers to dispersal within organs or between organs.

The histopathological changes induced by the larvae did not differ qualitatively between the two mouse strains. Foci of necrosis and attendant inflammation were observed in both and the inflammatory response was comparable to that previously described (Bindseil, 1981). Heavy infiltration of granulocytes and macrophages as well as necrosis was observed in both strains but the kinetics of these hepatic histopathological changes differed between the susceptible and resistant hosts. The earlier severe hepatic inflammatory reaction on day 4 p.i. in the resistant CBA/Ca mice coincided with the low numbers of larvae successfully migrating from the liver to the lungs whereas the more delayed severe inflammatory reaction on day 6 p.i. in the susceptible C57BL/6j mice occurred when the majority of the larvae had already migrated to the lungs. In the CBA/Ca mice, the resolution of the necrosis and inflammation was more rapid following the reduction of larval numbers.

Therefore, it appears that CBA/Ca mice handled the parasitic insult more effectively and economically in terms of responding earlier to restrict the same degree of larval invasion, and effecting more rapid tissue recovery without triggering additional inflammation. Day 4 p.i. has played a prominent role in earlier work on *Ascaris* infections. Larvae were encapsulated on day 4 p.i. onwards by inflammatory cells in guinea pigs immunized against *A. lumbricoides* (Soulsby, 1957) with consequent reduced larval numbers noted in the lungs (Soulsby, 1957; Khoury *et al.* 1977). Furthermore, neither little inhibition in *A. suum*-infected host growth nor a rise of eosinophilia was shown to be evident before this time in guinea pigs (Fallis, 1948; Soulsby, 1957). Therefore, day 4 p.i. has previously been considered a key time-point in effecting protection against *Ascaris* infection.

While it has been proposed that the day 4 p.i. inflammatory reaction in the liver of guinea pigs is induced by a larval moult in this organ (Taffs, 1968), L3 larvae have been observed in *Ascaris* ova (Geenen *et al.* 1999) and ecdysis has been shown not to occur in the liver (Fagerholm *et al.* 2000). Excretory-secretory products capable of inducing a response are continuously produced by the metabolic activity of larvae during migration (Guerrero and Silverman, 1969; Stromberg, 1979) and have been shown to differ depending on their developmental stage (Kennedy and Qureshi, 1986). The ES-antigens are considered targets for responses during larval migration and the temporal difference observed in hepatic inflammatory response in the current study may be due to restricted responses in the susceptible C57BL/6j to particular ES-antigens secreted early in hepatic tissue invasion. The *in vitro* response of previously infected NIH mice to *A. suum* ES-antigens has been observed (Kennedy and Qureshi, 1986) and a lack of responsiveness to the 14 000 MW component of both L2 and L3/L4 ES was noted. Interestingly, the NIH strain has been assigned since to the intermediate susceptibility category when infected with *A. suum* (Lewis *et al.* 2006). However, whether CBA/Ca mice respond to this particular ES-antigen has yet to be investigated and further investigation of the role of the liver in impeding migration in the mouse model will be required.

Previous evidence for an innate response accounting for resistance to *A. suum* infection in mice, coupled with a temporal difference in the hepatic cellular response observed in this study, indicate that examining the innate liver response to infection will be a fruitful line of enquiry for future work. As discussed by Holland (2009), we are now confident that we have a model of differential hepatic inflammation that is suitable for examining potential mechanisms of resistance and susceptibility in the liver. Future work will mainly focus on sentinel cells of the hepatic innate immune system and levels of cytokines and chemokines produced during infection, which may explain the temporal difference between strains in the hepatic inflammatory response observed.

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