Successful intestinal *Echinococcus multilocularis* oncosphere invasion and subsequent hepatic metacestode establishment in resistant RccHan™:WIST rats after pharmacological immunosuppression

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SUMMARY

Susceptibility/resistance to larval *Echinococcus multilocularis* infection varies greatly depending on host species and strains. Whereas several mice strains and non-human primates are highly susceptible to alveolar echinococcosis, rats and most of humans are considered as more resistant. In this study, we aimed to elucidate factors responsible for host resistance in rats (Experiments A–D). (A) The parasite establishment was not observed in immunocompetent Wistar rats orally inoculated with sodium hypochlorite resistant eggs with/without pig bile, or activated/non-activated oncospheres (NAO). Peritoneal inoculation with NAO or metacestode tissue allowed the parasite establishment in rats. (B) T-cell-deficient athymic nude rats showed complete resistance against the metacestode establishment after oral inoculation with parasite eggs. This finding suggests that T-cell-independent parasite clearance occurred in the animals during early phase of the parasite invasion. Finally, Wistar rats that received pharmacological immunosuppression using either dexamethasone (DMS) alone or methotrexate (MTX) i.p. alone or a combination of these compounds were orally inoculated with the parasite's eggs. As a result (D), successful establishment of metacestode with protoscoleces was observed in all 3 rats treated with DMS (s.c.) alone or in all 6 rats treated with DMS (s.c.) plus MTX but not in 8 rats with MTX alone, suggesting that factors affected by DMS treatment are responsible to regulate the parasite invasion and establishment.

Key words: alveolar echinococcosis, *Echinococcus multilocularis*, immunosuppression, metacestode, rat, resistance.

INTRODUCTION

Human alveolar echinococcosis (AE) is caused by the metacestode *Echinococcus multilocularis*. The parasite develops mainly in the liver as a tumour-like infiltrative mass that may cause the patient's death if not treated generally (Eckert *et al*. 2011). The disease is characterized by a long asymptomatic period with a slow but progressive metacestode development.

The infective stage of *E. multilocularis* to intermediate hosts including humans is the viable egg, which maturated in the gravid proglottid (Thompson, 1995). Each egg consists of an outer layer called embryophore and an oncosphere membrane surrounding a dormant oncosphere. When a viable egg is ingested by a host, embryophore disaggregation and subsequent oncosphere activation occur in the digestive tract triggered by temperature increase, pH decrease and the action of digestive enzymes. Although *E. multilocularis* eggs can hatch and develop in extra-intestinal locations (Thompson, 1995; Federer *et al*. 2015), features of the intestinal environment, e.g. bile salt composition, could contribute to differences in oncosphere activation in different intermediate host species. The activated oncospheres (AO) invade into the intestinal mucosa, and then move to predilection sites mainly in the liver tissues. Once established there, the parasite undergoes post-oncospheral development, leading to massive metacestode development with protoscolex formation in a susceptible intermediate host.

Susceptibility/resistance to larval *E. multilocularis* infection and disease development varies depending on the affected host species and strains. Despite sero-epidemiological data suggesting that humans are often exposed to *E. multilocularis* eggs in endemic

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areas, the incidence of AE is much lower than expected (Gottstein *et al*. 2001, 2015; Vuitton, 2003). These findings suggest that humans, at least the majority of population, are assumed to be basically resistant to AE infection. Monkeys including non-human primates have shown to be highly susceptible for AE with high mortality and morbidity in zoos (Deplazes and Eckert, 2001; Rehmann *et al*. 2003; Umhang *et al*. 2013). Meanwhile, pigs are considered as aberrant hosts, which seem less susceptible for parasite development after successful oncosphere invasion and establishment in the liver (Kamiya *et al*. 1987; Deplazes *et al*. 2005).

With regard to rodents, high susceptibility to *E. multilocularis* infection and aggressive metacestode development are observed in suitable intermediate hosts such as voles (Arvicolidae) (Obayashi *et al*. 1971; Woolsey *et al*. 2015*a*, *b*) and some inbred strains of mice (e.g. C57BL/6J, AKR, BALB/c) (Kamiya, 1972; Vuitton, 2003; Matsumoto *et al*. 2010). On the other hand, the brown rat (*Rattus norvegicus*) is considered highly resistant to larval *E. multilocularis* in nature (Rausch, 1995). To our knowledge, there is only one report available on natural infection with larval *E. multilocularis* in wild brown rats (Okamoto *et al*. 1992). This paper reports that 2 rats, out of 42 examined, harboured the fertile metacestodes with protoscoleces. With regard to experimental studies, Webster and Cameron (1961) reported that none of 5 rats showed establishment of *E. multilocularis* metacestode after oral inoculation with 0·5 mL of a suspension of gravid worm segments and eggs. In contrast, a steady growth occurs in laboratory rats (Wistar) upon inoculation of metacestode material via intraperitoneal or intrahepatic route (Inceboz *et al*. 2007; Li *et al*. 2011; Yamashita *et al*. 2013). Considering these findings in rats, host resistance against early phase of *E. multilocularis* infection (invasion and establishment of oncospheres in the host tissues) seems regulated by factor(s) different from those involved in resistance against post-oncospheral metacestode development.

So far, little is known about key factor(s) that regulate *E. multilocularis* oncosphere activation, invasion and subsequent establishment in resistant hosts such as rats and humans. In this study, we aimed to elucidate factors involved in host resistance in rats by using various forms of the parasite inoculate and host animals with different immunological states.

MATERIALS AND METHODS

Parasite material: eggs and oncospheres

Echinococcus multilocularis eggs were obtained from naturally infected foxes during the Swiss hunting seasons 2010–2014. Adult parasites were harvested from the small intestines of the foxes as described by Hofer *et al*. (2000). Subsequently, *E. multilocularis* eggs were collected after squashing and filtering the worms through 41 μm followed by 21 μm meshes (Lanz-Anliker AG, Switzerland) before being kept at 4 °C in PBS with 100 IU penicillin, 100 μg streptomycin and 0·25 μg amphotericin B (Life Technologies, Switzerland) for a maximum of 7 months until use. Integrity (maturity) of eggs was assessed by the sodium hypochlorite resistance test (Deplazes *et al*. 2005). The amount of mature eggs administrated to animals was adjusted accordingly. Non-activated oncospheres (NAO; free of embryophore but surrounded by the oncosphere membrane) and oncospheres after artificial enzymatic activation (AO) were prepared as previously described (Deplazes and Gottstein, 1991). Viability of AO was confirmed by microscopic observation of mobility of hatched oncospheres. Mature eggs, NAO and AO were inoculated orally to animals suspended in 0·4–1 mL (for rats) or 0·2–0·4 mL (for mice) of PBS. For intraperitoneal inoculation to animals, NAO were suspended in 0·25 mL (for rats) or 0·2 mL (for mice) of sterile phosphate-buffered saline (PBS).

Metacestodes

Fresh *E. multilocularis* metacestode material was collected from the peritoneal cavity of gerbils (*Meriones unguiculatus*) after euthanasia, which were experimentally infected with the KF5 isolate (Gottstein *et al*. 1992). This material was subsequently sieved through a 500 μm mesh and washed several times with sterile PBS. Metacestode tissue was suspended in 0·15 mL (for rats) or 0·1 mL (for mice) of sterile PBS and immediately intraperitoneally inoculated to animals.

Experimental animals

All animal experiments described in this paper were authorized by the Cantonal Veterinary Office of Zurich, Switzerland (permission no. 33/2009; 194/2010; 192/2011; 294/2014) prior to the start of the studies. RjHan_WI (Wistar) rats (1–2 month of age) were purchased from Janvier Labs (France), RccHan™:WIST (Wistar) rats (2–3 month of age) and Hsd:RH-Foxn1^{rnu} (athymic nude) rats (4–5 month of age) used in the present study were purchased from Harlan Laboratories (Switzerland). C57BL/6 mice (2–3 month of age) were obtained from Charles River Laboratories (Germany) and were used as *in vivo* controls for infectivity of utilized materials and were infected along with the rats in each experiment. The number of animals in each experimental group is shown in Tables 1–3.

Experimental design

This study involved 4 different animal experiments (Experiments A–D). Experiment A was based on

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Table 1. Establishment of *Echinococcus multilocularis* metacestodes in RjHan_WI (Wistar) rats after p.o./i.p. inoculation with different forms of the parasite inoculate

Experiment	Group	Animal species/ type (strain)	Form of inoculum (amount)	Route of inoculation	Weeks p.i. at autopsy	No. of animals (males)	Animals with parasite establishment (location)
Exp. A	AM1	mouse (C57BL/6)	eggs (6000)	p.o.	4	4	2 (liver)
	AM2		metacestode tissues	i.p.	24	3	3[a] (abdominal cavity)
	AM3		NAO (10 000)	i.p.	24	3	3[a] (abdominal cavity)
	AR1	Wistar rat (RjHan_WI)	eggs (10 000)	p.o.	24	6	0[b]
	AR2		eggs with pig bile (10 000)	p.o.	24	6	0[b]
	AR3		NAO (10 000)	p.o.	24	6	0[b]
	AR4		AO (10 000)	p.o.	24	6	0[b]
	AR5		metacestode tissues	i.p.	24	3	3[a] (abdominal cavity)
	AR6		NAO (10 000)	i.p.	24	3	2[a] (abdominal cavity, s.c.)

NAO, non-activated oncospheres; AO, activated oncospheres.
[a] positive PCR (Stieger *et al*. 2002).
[b] negative PCR (Stieger *et al*. 2002).

the hypothesis that the gastrointestinal environment of rats or mice could affect oncosphere hatching, activation and subsequent invasion and establishment in host tissues. Therefore, 4 different types of inoculates were tested for oral administration: (1) mature eggs; (2) mature eggs with pig bile; (3) NAO; (4) AO. Additionally, metacestode and NAO were inoculated intraperitoneally to compare parasite establishment in the animals without exposing them to the gastrointestinal environment of host animals. In the following 3 experiments, we analysed whether the immunological status of rats influences the successful invasion and establishment in host tissues by larval *E. multilocularis*. In order to clarify this point, T-cell deficient athymic nude (Experiment B) and pharmacologically immunosuppressed rats (Experiments C and D) were infected and compared with immunocompetent rats.

Experiment A: Parasite establishment in animals after per oral/intraperitoneal [per os (p.o.)/intraperitoneal (i.p.)] inoculation with different forms of parasite inoculates

Design of Experiment A is summarized in Table 1. Briefly, RjHan_WI (Wistar) rats were orally inoculated with a high infection dose of 10 000 mature *E. multilocularis* eggs per animal without (group AR1) or with (group AR2) the addition of 10% pig bile. The other two groups were orally inoculated with 10 000 NAO (group AR3) or AO (group AR4). As a control, C57BL/6 mice received an oral dose of 6000 *E. multilocularis* eggs (group AM1). Intraperitoneal inoculations were carried out in Wistar rats with metacestode tissue (group AR5), or with 10 000 NAO (group AR6). Metacestode tissues (group AM2) or 10 000 NAO (group AM3) were intraperitoneally inoculated to C57BL/6 mice as controls. The animals were autopsied at 4 weeks (group AM1) or 24 weeks (all the other groups) post-inoculation (p.i.) to examine parasite establishment.

Experiment B: Parasite establishment in athymic nude rats

Design of Experiment B is summarized in Table 2. Briefly, T-cell-deficient Hsd:RH-Foxn1^{rnu} (athymic nude) rats were orally inoculated with a moderate dose of 900 mature *E. multilocularis* eggs. As a control, the same amount of parasite eggs was given to C57BL/6 mice. C57BL/6 mice (group BM1) necropsy was performed 12 weeks p.i., while nude rats were examined for the parasite establishment at 6 weeks (group BR1), 12 weeks (group BR2), 18 weeks (group BR3) and 24 weeks (group BR4) p.i.

Experiment C: Parasite establishment in rats with pharmacological immunosuppression (part 1)

Design of Experiment C is summarized in Table 3. Two weeks prior to oral inoculation with a moderate dose of 1000 mature *E. multilocularis* eggs, RccHan™: WIST (Wistar) rats (group CR2) were subjected to pharmacological immunosuppression using a combination of a weekly intraperitoneal injection with methotrexate (MTX; Pfizer, Germany) (0·6 mg kg^{-1} body weight) and daily administration of dexamethasone (DMS; Galepharm AG, Switzerland) (2 mg kg^{-1} body weight) given in drinking water. This immunosuppressive treatment was continued

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Table 2. Establishment of *Echinococcus multilocularis* metacestodes in Hsd:RH-Foxn1^{rnu} (athymic nude) rats. Inoculation dose (p.o.) of each animal consists of 900 *E. multilocularis* eggs

Experiment	Group	Animal species/type (stain)	Weeks p.i. at autopsy	No. of animals (males)	No. of animals with parasite establishment (location)
Exp. B	BM1	mouse (C57BL/6)	12	2	2 (liver)
	BR1	nude rat (Hsd:RH-Foxn1^{rnu})	6	3	0[a]
	BR2		12	4	0[a]
	BR3		18	3	0[a]
	BR4		24	20	0[a]

[a] negative PCR (Stieger *et al*. 2002), 3 liver samples tested.

until 6 weeks after oral egg inoculation. As immunocompetent control groups, C57BL/6 mice (group CM1) and Wistar rats (group CR1) were used without immunosuppressive treatment. All animals were orally inoculated with 1000 mature *E. multilocularis* eggs. Necropsy was performed at 12 weeks (group CM1) or 24 weeks (groups CR1 and CR2) p.i.

Experiment D: Parasite establishment in rats with pharmacological immunosuppression (part 2)

Design of Experiment D is summarized in Table 3. Seventeen days prior to oral inoculation with 1000 mature *E. multilocularis* eggs, immunosuppressive treatment of the 2 month aged female or male rats started using either DMS (Dexafort, MSD Animal Health, Germany) alone (group DR2), MTX alone (0·6 mg kg^{-1} body weight/week, i.p.) (group DR3) or a combination of both compounds (group DR4). Exact dosage was controlled by individual weight measurements. The treatment with DMS was initiated with the first injection dose of 1·5 mg animal^{-1} subcutaneously (s.c.) as described previously (Ciesielski *et al*. 1998; Habib and El Garhy, 2002). However, we used a reduced dose of DMS (0·75 mg animal^{-1}, s.c.) for the second and third injections because obvious weight losses were observed after the first application of DMS in some of the animals in groups DR2 and DR4. Moreover, DMS treatment was terminated after the third application, which was given 9 days prior to the egg inoculation (with the exception of one male rat in group DR2 and 1 male rat in DR4 that were inoculated 15 days after last treatment). Despite these modifications in use of DMS, 5 rats in group DR2 and 2 rats in group DR4 were excluded from experiments due to weight losses by over 20% according to previously defined termination criteria. On the other hand, MTX treatment was continued as scheduled until 4 weeks p.i. in groups DR3 and DR4. Immunocompetent control groups consisted of C57BL/6 mice (group DM1) and Wistar rats (group DR1) without immunosuppressive treatment. All animals were orally inoculated with 1000 mature *E. multilocularis* eggs. Necropsy was carried out 10–11 weeks p.i. for all the animals.

Macroscopical and histological examinations

At necropsy, developed metacestode samples were collected and fertility was evaluated by direct visualization of protoscoleces under a light microscope. In addition, some metacestode material was fixed in 10% buffered formalin. After fixation, serial paraffin sections were made and stained with haematoxylin and eosin before examined microscopically.

PCR for the detection of E. multilocularis

Liver samples were randomly collected from several rats with no macroscopically visible metacestode tissue. These liver samples were subjected for PCR detecting *E. multilocularis* mitochondrial 12S rRNA gene (Stieger *et al*. 2002) in order to examine the presence/absence of the parasite in the tissues with no macroscopically clear metacestode establishment. For this purpose, DNA was extracted from the collected tissues using a commercial kit (QIAamp DNA mini kit, QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions (tissue protocol).

RESULTS

Parasite establishment in animals after p.o./i.p. inoculation with different forms of parasite inoculates

The result of Experiment A is shown in Table 1. No parasite development could be macroscopically detected in any of the rat groups that received oral parasite inoculates: mature eggs (group AR1) or eggs with pig bile (AR2), or non-activated (AR3) or activated (AR4) oncospheres. Negative results of the PCR detecting *E. multilocularis* in the liver tissue samples confirmed the absence of the parasite in all tested rats. In contrast, 2 out of 4 mice orally inoculated with mature eggs (group AM1) developed macroscopically visible metacestodes in the liver, 4 weeks p.i.

On the other hand, all of the mice (group AM2) and rats (group AR5) that were intraperitoneally inoculated with metacestode tissue developed metacestode masses. Additionally, all 3 mice (group AM3) and 2 out of 3 rats (group AR6) that were intraperitoneally

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Table 3. Establishment of *Echinococcus multilocularis* metacestodes in RccHan™:WIST (Wistar) rats with/without pharmacological immunosuppressive treatment. Inoculation dose (p.o.) of each animal consists of 1000 *E. multilocularis* eggs

Experiment	Group	Animal species/type (strain)	Immunosuppressive treatment (route)	Weeks p.i. at autopsy	No. of animals and gender (m/f)	No. of animals with parasite establishment (location)
	CM1	mouse (C57BL/6)	n/a	12	2 (m)	2 (liver)
Exp. C	CR1	Wistar rat	n/a	24	6 (m)	0[a]
	CR2	(RccHan™: WIST)	DMS (p.o.) + MTX (i.p.)	24	6 (m)	2[b] (liver)
	DM1	mouse (C57BL/6)	n/a	10–11	2 (m), 2 (f)	2 (liver)
	DR1		n/a	10–11	4 (m), 4 (f)	0
Exp. D	DR2	Wistar rat	DMS (s.c.)	10–11	3 (m)	3 (liver)
	DR3	(RccHan™:	MTX (i.p.)	10–11	4 (m), 4 (f)	0
	DR4	WIST)	DMS (s.c.) + MTX (i.p.)	10–11	3 (m), 3 (f)	6 (liver)

DMS, dexamethasone treatment; MTX, methotrexate treatment; n/a, not applied.
[a] negative PCR (Stieger *et al*. 2002).
[b] positive PCR (Stieger *et al*. 2002).

inoculated with NAO showed metacestode establishment. The metacestodes were found in the peritoneal cavities of these animals except 1 rat, which harboured the parasite subcutaneously at the site of inoculation. For all the animals, establishment of *E. multilocularis* was confirmed by histological observation of the metacestode samples. Furthermore, the presence of the parasite in the samples obtained from the rats was confirmed by specific PCR.

Parasite establishment in athymic nude rats

The result of Experiment B is shown in Table 2. None of the nude rats inoculated with mature *E. multilocularis* eggs (groups BR1–BR4) showed the parasite establishment on any of the scheduled necropsy dates (at 6, 12, 18 and 24 weeks p.i.). The results of the PCR specific for *E. multilocularis* were negative for 3 liver samples collected from the rats. On the other hand, the simultaneous inoculation of mice (group BM1) with the parasite eggs resulted in the parasite establishment in the liver.

Parasite establishment in rats with pharmacological immunosuppression

The result of Experiment C is shown in Table 3. The untreated immunocompetent control rats (group CR1) orally inoculated with *E. multilocularis* eggs showed no evidence for the parasite establishment. Specific PCR using randomly selected liver tissue was negative in all 10 analysed samples. On the other hand, 2 out of 6 rats that were pharmacologically immunosuppressed with DMS (p.o.) plus MTX (group CR2) showed *E. multilocularis* metacestode development in the liver as confirmed by the presence of protoscoleces in morphological observation (Fig. 1). One animal exhibited massive growth of the metacestode (Fig. 1A and B) whereas the other had a more discrete lesion (Fig. 1C and D). In the remaining 4 rats of this group, *E. multilocularis* establishment could not be evidenced. Moreover, specific PCR carried out with randomly-selected liver tissues resulted all negative. The simultaneous oral inoculation of C57BL/6 mice (group CM1) with the parasite eggs performed as a control resulted in the parasite establishment similar to Experiments A and B.

The result of Experiment D is shown in Table 3. Again, the untreated, immunocompetent rats (group DR1) had no parasite establishment after oral inoculation with *E. multilocularis* eggs. On the other hand, all the remaining rats that received immunosuppressive treatment with DMS alone (group DR2) or with DMS plus MTX (group DR4) exhibited metacestode burdens in the liver at the time of necropsy. The total liver weight of each animal with metacestode establishment was highly variable and up to 8-fold higher than the average weights of the non-infected livers (Fig. 2). In contrast, none of the rats treated with MTX alone (group DR3) showed parasite lesions. The simultaneous inoculation of C57BL/6 mice (group DM1) with parasite eggs performed as a control resulted in the parasite establishment as found in Experiments A–C. Animals of both genders were used in this experiment except for DR2 group, in which all the females had to be excluded due to weight loss. There was no gender-dependent difference in each experimental group in terms of the parasite establishment.

DISCUSSION

Most of the human population is considered rather resistant to larval *E. multilocularis* infections (Gottstein

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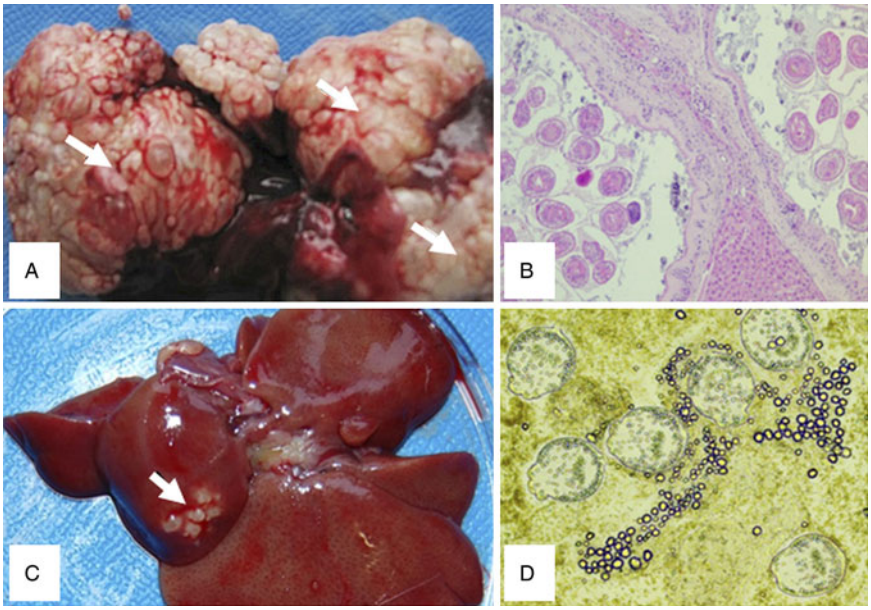

Fig. 1. Macroscopical and microscopical views of *Echinococcus multilocularis* metacestodes (white arrows) in the liver, which were found in the 2 pharmacologically (with dexamethasone and methotrexate) immunosuppressed (RccHan™: WIST) Wistar rats ($n = 6$) belonging to group CR2 in Experiment C, 24 weeks p.i. with 1000 *E. multilocularis* eggs. One of these animals showed massive growth of the metacestode (A) with mature protoscoleces confirmed in a histological section of the parasite (B; H&E stain). The other animal had a more discrete lesion (C) containing protoscoleces, which were observed in a smear sample of the metacestode material (D).

et al. 2015). This is reflected by mass screening data in endemic areas where a higher number of seropositive patients was found compared with the number of established infections (Bresson-Hadni *et al*. 1994; Romig *et al*. 1999; Gottstein *et al*. 2001). Among rodents, rats are also considered resistant to *E. multilocularis* egg infection in nature (Rausch, 1995). In the present study, we tried to elucidate factors involved in host resistance against *E. multilocularis* using rat models. Firstly (Experiment A), it was examined whether differences in oncosphere conditions could be responsible for different resistance/susceptibility against the larval infection between rats and mice. As a result, we found that the parasite was not detected in any of the rat groups inoculated orally with the parasite's eggs plus pig bile, NAO, nor AO. Based on these results, it is concluded that different gastrointestinal factors in mice or rats do not account for a successful hatching of the oncospheres and thus are probably not responsible for host resistance against the parasite infection in rats. Interestingly, on the other hand, intraperitoneal inoculation with NAO resulted in the successful establishment of the metacestodes in 2 of the 3 immunocompetent rats, demonstrating that *E. multilocularis* oncospheres were able to develop into metacestodes in the peritoneal cavity or subcutaneously of a naturally resistant host. In addition, this finding suggests the intestinal activation is not essential for the oncospheres to establish and develop as metacestodes. This is consistent with the results of the highly sensitive viability test for *E. multilocularis* using s.c. inoculation of NAO in mice (Federer *et al*. 2015) and our observation that NAO started vesiculation when they were co-cultured *in vitro* with a mammalian cell line, 3T3 (J. Matsumoto, unpublished observation).

The following experiment (Experiment B) was performed to examine the importance of T-cell-mediated immunity against invasion and subsequent establishment by *E. multilocularis* oncospheres using athymic nude rats. As a result, no parasite lesion was found in any of the animals examined at 6–24 weeks p.i. This finding suggests that T-cell-independent parasite killing occurred in the animals by this time point after the egg ingestion, although the exact mechanism of the parasite elimination is to be clarified. In this context, it is worth pointing out that athymic nude rats (rnu/rnu) investigated by Reynolds *et al*. (1982) are known to have a higher level of natural killer cell activity than euthymic rats, which are mainly attributed to an increased proportion of effector cells. Since the characteristics of athymic nude rats used in Experiment B (animal's datasheet available as pdf-file on the following web page: old.harlan.com/download.axd/ff11af708f4744939bd43e98f79dc81a.pdf?d=RMS0109-US-EN-01-PS-30_AthymicNudeRat; reference taken on 10/01/2016) indicate likewise an increased natural killer cell and macrophage population, this cell population could have been associated with the parasite clearance in nude rats during an early phase of this experimental infection. It should be noted that different kinds of immune responses are required to eliminate different development stages of the parasite. In

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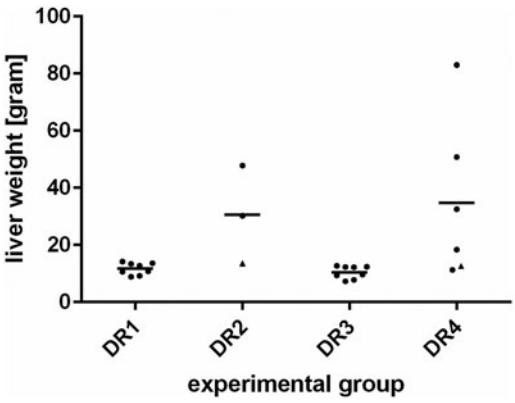

Fig. 2. Individual liver weights of (RccHan™:WIST) Wistar rats (Experiment D), 10–11 weeks after oral inoculation with 1000 *E. multilocularis* eggs (dots: 6 days; triangles: 15 days after last immunosuppressant treatment). DR1 = non-immunosuppressed control ($n = 8$); DR2 = immunosuppressed with dexamethasone alone ($n = 3$); DR3 = immunosuppressed with methotrexate alone ($n = 8$); DR4 = immunosuppressed with dexamethasone plus methotrexate ($n = 6$). Lines indicate mean values of the groups. Rats of group DR1 and DR3 showed no parasite growth in the liver.

contrast to our finding, it has been elucidated that the importance of T-cell responses during the course of AE evoking a Th-1 dominated immune response during the initial liver invasion (Baron and Tanner, 1976; Gottstein and Hemphill, 2008). Furthermore, a more recent experimental study hypothesized that the initial Th-1 activation was induced by a T-cell immunomodulatory protein (EmTIP) present in excretory/secretory products from cultivated primary cells of the parasite (Nono *et al*. 2014). Severe combined immune-deficient (SCID) mice, which lack functional B- and T-lymphocytes, were highly susceptible to *E. multilocularis* metacestode tissue growth (Playford *et al*. 1993), indicating that T-cell-dependent immune mechanisms evolve after oncosphere invasion and settlement in the liver tissue. On the other hand, innate immune mechanisms have been hypothesized to be responsible for an increase or decrease of susceptibility to AE in experimental animals (Vuitton, 2003; Gottstein *et al*. 2015).

In the third experiment (Experiment C), pharmacologically immunosuppressed rats were subjected to *E. multilocularis* infection. Two out of the 6 animals that had been administered orally with parasite eggs showed mature metacestode development with protoscolex formation in the liver at 24 weeks p.i. This is the first report on a successful establishment and fertile development of *E. multilocularis* metacestode in rats experimentally inoculated with the parasite eggs. The reason why no parasite establishment was observed in the 4 remaining animals could be attributed to insufficient immunosuppression as DMS was given to the animals by drinking water.

The results of the subsequent experiment (Experiment D) using rats immunosuppressed with either DMS (s.c.) or MTX or a combination of these components confirmed that pharmacologically immunosuppressed rats lost their resistance against *E. multilocularis* egg inoculation when treated with DMS but not MTX alone. This suggests that factors affected by DMS treatment are responsible to regulate parasite invasion, establishment and development of *E. multilocularis* oncospheres and metacestodes. DMS inhibits innate and adaptive immunity on different levels (Zen *et al*. 2011) whereas MTX is a rather specific immunosuppressive agent with profound effects on lymphocytes and weak effects on monocytes (Wessels *et al*. 2008). In addition, we should consider other possibilities that DMS has a direct or indirect effect on unknown factors other than host immune response to the parasite. By comparing the effects on immunological functions in rats induced by DMS and MTX, candidate factors could be clarified that are important for host resistance during early phase of *E. multilocularis* infection in the rat models. In this experiment, we used animals of both genders and found no gender-dependent difference in each group regarding the parasite establishment. Thus, the host resistance against the parasite invasion and establishment seem unrelated to the gender of host animals.

It was observed in the course of Experiment D that 5 female rats (2–3 month of age, weight range 149–156 g) and additionally 2 male rats (2–3 month of age, weights 196 and 199 g) exhibit weight losses by over 20% after DMS treatment and had to be excluded from the experiment. Further rat experiments should therefore consider the initial weights of animals to avoid massive weight losses after DMS-treatment.

With regard to human cases, an increased number of AE cases have been reported recently, who received immunosuppressive treatment for organ transplantation, malignant- and chronic inflammatory diseases (Gruener *et al*. 2008; Kayacan *et al*. 2008; Gaultier *et al*. 2009; Geyer *et al*. 2011; Kern *et al*. 2011; Weiner *et al*. 2011 and Dentan *et al*. 2012). Vuitton *et al*. (2015) pointed that AE patients with immunosuppression showed significantly higher progression of the disease compared with the non-immunosuppressed. Thus, acquired therapeutic immunosuppression is considered the main factor for AE occurrence and also its fast progression (Vuitton *et al*. 2015). It is getting more and more relevant to clarify how pharmacological immunosuppression affects invasion and subsequent establishment processes of the parasite in naturally resistant hosts such as rats and non-immunosuppressed humans.

In summary, here we report for the first time that establishment and development of *E. multilocularis*

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metacestodes with protoscolex formation occurs after egg inoculation in naturally resistant rats followed immunosuppression by using proper compounds such as DMS. This animal model provides us with an option to further analyse key factors responsible for host resistance against larval *E. multilocularis* infection.

CONFLICT OF INTEREST

The authors have declared that they have no competing interests.

AUTHORS' CONTRIBUTION

M. T. A. F., D. J. and A. S. performed the experiments. P. D. and A. S. have contributed to designing the study and R. M. E. has assisted during the animal experiments. All authors contributed to the writing of the manuscript and approved the final version.

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