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ORIGINAL PAPER

**Bruno Gottstein · Wen Juan Dai · Mirjam Walker
Marianne Stettler · Norbert Müller · Andrew Hemphill**

An intact laminated layer is important for the establishment of secondary *Echinococcus multilocularis* infection

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Abstract *Echinococcus multilocularis* causes alveolar echinococcosis primarily in rodents, but also in humans where it represents one of the most lethal helmintic infections. We used a susceptible mouse (C57BL/6) model to demonstrate failure in controlling secondary infection with the *E. multilocularis* metacestode, even when performed at the lowest possible infection dose. This was achieved by intraperitoneal or intrahepatic inoculation of a single parasite vesicle. In secondary infections, the primary physical barrier between the parasite and the host is constituted by the acellular laminated layer (LL), which is predominantly composed of high-molecular-weight glycans and surrounds the entire metacestode. Only those metacestode structures which exhibited an intact LL were successful in establishing infection, whereas metacestodes which were punctured – thus exhibiting an opened LL and thereby an accessible germinal layer – were no longer infective. Conversely, both types of vesicle survived in vivo maintenance, as assessed by RT-PCR based upon II/3 gene expression. In consequence, the encapsulating LL appears to be one of the key factors that mediates survival and successful proliferation of the parasite metacestode in vivo.

Introduction

Alveolar echinococcosis (AE) is a severe hepatic disorder caused by the infection with the metacestode stage of a small fox tapeworm, *Echinococcus multilocularis*. Despite the public health importance of AE in areas such as Central Europe, Alaska and China, we still lack detailed knowledge on the parasite survival strategy, on parasite–host interactions and on the immunological control of *E. multilocularis* infection. So far, most experimental studies on AE have been performed in the laboratory mouse model (reviewed in Gottstein 2001). Commonly, secondary infections are initiated by intraperitoneal (i.p.) or intrahepatic (i.h.) inoculation of metacestode material, in most cases represented either by a suspension of metacestode vesicles, clusters of vesicles or appropriately small pieces of metacestode tissue. Morphologically and functionally, the smallest metacestode unit consists of a single fluid-filled vesicle, harboring an inner, germinal layer representing the actual live parasite tissue and an outer, acellular laminated layer (LL) surrounding the entire metacestode (Ingold et al. 1999). Parasite proliferation is primarily achieved by exogenous budding from such vesicles, which subsequently results in mass expansion and metastasis formation into the adjacent host tissues.

Periparasitically, the development of the metacestode is associated with a massive granulomatous reaction and an intense fibrogenesis (Bories et al. 1996). Progressively, both parasite-specific humoral and cell-mediated immune responses are established (reviewed by Gottstein and Hemphill 1997) and the latter seems to play a crucial role in the control of *E. multilocularis* infections (Bresson-Hadni et al. 1990; Gottstein et al. 1994; Emery et al. 1996; Amiot et al. 1999). A recent study, however, demonstrated that humoral immunity may also be important in the control of parasite growth (Dai et al. 2001). It was documented that the LL-associated antigen Em2(G11; Deplazes and Gottstein 1991) induces an IgG response independent of $\alpha\beta+$ and CD4+ T cells, an event which could be linked to the restricted T-cell activation following *E. multilocularis* infection (Gottstein et al. 1994), lack of avidity maturation of some antibody populations (Dai et al. 2001) and thus to the inability of

B. Gottstein (✉) · M. Walker · M. Stettler · N. Müller
A. Hemphill
Institute of Parasitology, University of Berne,
Länggass-Strasse 122, 3012 Berne, Switzerland
E-mail: bruno.gottstein@ipa.unibe.ch
Fax: +41-31-6312622

W.J. Dai
Clinic for Rheumatology
and Clinical Immunology/Allergology,
University Hospital, Inselspital,
3010 Bern, Switzerland

immunocompetent hosts to control the infection by immunological means. Such studies have strongly suggested that not only the Em2(G11) antigen, but also the LL by itself plays an important role in protecting the metacestode from host immune responses (Gottstein et al. 1992; Dai and Gottstein 1999; Dai et al.2001).

In the present study, we infected susceptible C57BL/6 mice – comparatively susceptible to a conventional infection dose of 100 vesicles – with the lowest possible structural infection dose, represented by one single *E. multilocularis* metacestode vesicle, in order to determine the outcome of single-vesicle infection in immunocompetent mice. Furthermore, single-vesicle infection using intact metacestodes was compared to infection with punctured metacestodes. A punctured vesicle allows the host to directly access the interior germinal layer of the parasite. Thus, this study provides additional information on how the LL contributes to the protection of the parasite against host effector mechanisms.

Materials and methods

Maintenance of parasite and in vitro cultivation

Tissue culture reagents were purchased from Gibco (Basel, Switzerland). The cloned *Echinococcus multilocularis* isolate KF5 (Gottstein et al. 1992) was maintained by serial passage in gerbils (*Meriones unguiculatus*). In vitro culture of *E. multilocularis* metacestodes was carried out as described by Hemphill and Gottstein (1995). Briefly, parasite tissue was aseptically recovered from the peritoneal cavity of infected animals. The tissue pieces were cut into small tissue blocks (0.5 cm^3) and these were washed in Hank's balanced salt solution, before 6–12 tissue pieces were placed into 50 ml of culture medium (RPMI 1640 containing 12 mM Hepes, 10% fetal calf serum, 2 mM glutamine, 200 µg penicillin/ml, 200 µg streptomycin/ml, 0.50 µg fungizone/ml). Tissue blocks were kept in culture flask (75 cm^2) placed in an upright position at 37 °C, 5% CO_2, with medium changes every 2–4 days. Parasite vesicles produced from these primary tissue blocks were recovered by careful aspiration with a sterile pipette. Vesicles were subsequently washed in sterile RPMI 1640 and were either used for infection of mice, for production of vesicle fluid (VF) antigen, as described by Hemphill and Gottstein (1995), or for in vitro maintenance to assess survival of intact or punctured vesicles (see below).

Infection of mice

Female 6- to 10-week-old C57BL/6 mice were purchased from Biotechnology and Animal Breeding Division, Füllinsdorf, Switzerland. Animals were infected separately at two different doses (conventional/normal dose = 100 vesicles, low dose = 1 parasite vesicle only) and by different routes (i.p., i.h.). For conventional dose infections, approximately 100 vesicles were purified from in vitro cultures and resuspended in 100 µl of RPMI 1640. The intraperitoneal injections were performed using a 1.2×40 mm hypodermic needle. Control mice received 100 µl of RPMI 1640 only. Infection by i.h. was performed by surgical implantation of vesicles under a microsurgical microscope.

Infection with a single parasite vesicle was performed either with an intact vesicle (Fig. 1a) or with a vesicle of which the laminated layer had been carefully punctured in vitro under a surgical binocular, using a sterile 0.4×19 mm hypodermic needle (Fig. 1b/d). Vesicles were then injected i.p., using a 1.2×40 mm hypodermic needle. In order to confirm that the experimental inoculation was successful, the syringe was rinsed with medium and the fluid checked for the absence of the vesicle. Infection with a single vesicle by i.h. was performed by surgical inoculation, similar to normal-dose infection.

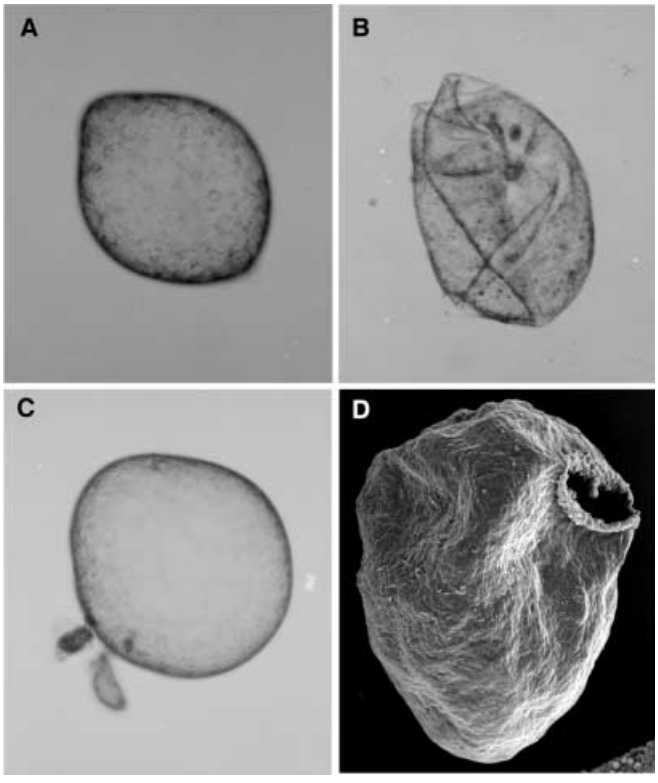

Fig. 1a–d Exemplary vesicles isolated from in vitro cultivated metacestodes, as used for infection of mice (diameter of a vesicles approximately 1 mm). **a** Intact vesicle, characterized by its fluid-filled bloated cystic form, indicating integrity of the laminated layer (LL). **b** Identical vesicle after in vitro puncturing with a 0.4×19 mm hypodermic needle. Basic structures are maintained, including an intact germinal layer attached to the LL, but loss of fluid is exhibited by the drooping appearance of the vesicle wall (see also **d**).**c** Vesicle as shown in **a**, but at an advanced proliferative stage with the presence of exogenous buds. Such buds may have already separated their germinal layer and LL from the mother cyst, thus being unaffected by puncturing. **d** Scanning electron micrograph of a punctured vesicle, demonstrating that the structural integrity of these metacestodes has largely been maintained

Assessment of infection

Infected mice were euthanized by CO_2. Peritoneal cavities and livers were carefully inspected for the presence of metacestodes. Peritoneal metacestodes were removed and the parasite weights were determined in each individual mouse. Livers were dissected and the lesion number was determined. Subsequently, liver tissue was fixed in 4% buffered formaldehyde solution, embedded in paraffin and histological sections were stained with hemalaun-eosin.

Detection of specific serum antibodies by ELISA

Sera were analyzed for the presence of antibodies directed against VF antigen by ELISA, as described by Gottstein et al. (1994) and Dai et al. (2001). ELISA wells were coated with VF antigen at a concentration of 5 µg protein/ml. This coating

concentration had been previously optimized by serial-dilution experiments, using positive sera obtained from another experiment (Dai et al. 2001).

Assessment of vesicle viability by RT-PCR

Intact and punctured vesicles were separately maintained in vitro for a time period of 14 days, in parallel to mouse infections using the same parasite materials. To prove the viability of parasite cells with punctured vesicles maintained in vitro (compared with non-punctured vesicles) RT-PCR was performed, based upon the expression of the II/3 gene (Felleisen and Gottstein 1994; Kern et al. 1995). Total RNA was extracted from approximately 40 intact or punctured vesicles, using the Trizol reagent system (GibcoBRL, Basel, Switzerland). Subsequently, residual genomic DNA was removed from the RNA by performing a 1-h incubation step at 37 °C in the presence of 1 unit of RQ1 RNase-free DNase (Promega, Madison, Wis., USA) and 1× M-MLV-RT buffer (Promega). After heat-inactivation (5 min at 95 °C) of the DNase, RNA extracts were subjected to cDNA synthesis, by applying a random oligo-hexamer primer/M-MLV-RT reaction, according to the manufacturer's instructions.

PCR was carried out in a 50 µl mixture containing 5 µl of 10× Gene AMP-PCR buffer (Applied Biosystems, Rotkreuz, Switzerland), 0.2 mM each dATP, dGTP and dCTP, 0.4 mM dUTP (Pharmacia, Dübendorf, Switzerland), 50 pmol each of gene II/3-specific forward RB3 (5′-GGAGGAACGATTGCAACGTATGG-3′) and reverse RB4 (5′-TTCTCACGCATTCTGCGAAGCTC-3′) primers; 1.25 units of AmpliTaq DNA polymerase (Applied Biosystems) and 0.5 units of heat-labile Uracil DNA glycosylase (Roche Diagnostics, Rotkreuz, Switzerland). PCR was done with the following temperature profile: 1 cycle of 120 s at 50 °C and 300 s at 95 °C, then 45 cycles of 30 s at 94 °C, 30 s at 66 °C and 120 s at 72 °C, with a final 15-min extension at 72 °C. Amplification reactions contained either cDNA equivalents from approximately four intact or punctured vesicles, or DNA from the positive control plasmid pG11 (kindly provided by R. Felleisen), containing a gene II/3 cDNA sequence determined by the primer RB3 and RB4 sequences (GenBank accession number U05573). Negative control reactions were done with total RNA samples which had been incubated as described above, but in the absence of M-MLV-RT. The 371 base pair (bp) amplification products were analyzed by electrophoresis in a 2% agarose gel (see Fig. 3).

Statistical methods

Comparisons were analyzed by Student's *t*-test using Microsoft Excel:mac 2001 software on a Macintosh G3 computer. Significances were set at $P < 0.05$.

Results

Infection with *Echinococcus multilocularis* metacestodes

The establishment of host cell-free *Echinococcus multilocularis* metacestode in vitro culture (Hemphill and Gottstein 1995) opened the possibility of infecting mice with a defined number of metacestodes. We wanted to know whether C57BL/6 mice can control an infection performed at the lowest possible dose (one vesicle) and to study whether the LL is an important factor in the establishment of secondary infection. Therefore, C57BL/6 mice (5–9 mice/group) were inoculated either with a single metacestode surrounded by an intact LL (Fig. 1a, c) or with a vesicle that had been punctured just prior to infection (Fig. 1b). Puncturing the vesicle did not dramatically affect the viability of the germinal layer, but provided direct host accessibility to the interior parasite compartment (Fig. 1d). Infections were performed either by i.p. or i.h. inoculations. Mice were sacrificed at 2 months post-infection (p.i.) and 4 months p.i., respectively, for analyzing parasite growth and host immune responses. In order to assess reproducibility under long-term infection conditions, the parasite weight was determined in two independent other groups sacrificed at 7 months p.i. and 8 months p.i., respectively.

Infection with either a single intact vesicle or with 100 vesicles resulted in progressive metacestode development and subsequent metastasis formation in all three groups of mice sacrificed at 4 months p.i. (Table 1). Identical results were obtained when infection was terminated at 2 months p.i. (data not shown). Total metacestode weight (including metastases) was assessed in i.p.-infected mice; and the parasite masses recovered after 4 months p.i. from mice infected with 100 vesicles were significantly higher than those from animals infected with a single vesicle. In i.h.-infected mice – due to the primary intrahepatic localization – the parasite growth had to be assessed by determining the lesion size/diameter in millimeters. The primary vesicles in i.p.-in-

Table 1 Infection of C57BL/6 mice with a single metacestode vesicle by intraperitoneal (i.p.) versus intrahepatic (i.h.) means, compared with the injection of a standard 100-vesicle suspension i.p. Infections were terminated at 4 months post-infection. *PW* Parasite weight: i.p. injection allowed the recovery of all i.p. metacestode tissue and the determination of the exact parasite weight. i.h. inoculation led to primary i.h. parasite localization. Metacestode masses were determined by average diameter of lesion. Individual metacestode lesions varied considerably in size (data not shown)

Mouse	i.p. injection of one vesicle		i.h. inoculation of one vesicle		i.p. injection of 100 vesicles (control)	
	PW (mg)	Location/metastasis	Parasite size/ lesion diameter	Location/metastasis	PW (mg)	Location/metastasis
1	39	One in peritoneum	7 mm	One in liver	9,850	Four in peritoneum
2	125	two in liver	2 mm	One in liver	380	Five in peritoneum
3	65	Three in peritoneum	5 mm	Two in liver, one in peritoneum	12,430	Three in peritoneum, one in liver
4	44	Two in peritoneum, one in liver	2 mm	One in peritoneum	5,760	Two in peritoneum, one in diaphragm
5	68	Four in peritoneum	3 mm	One in peritoneum	2,820	Two in peritoneum

fected mice formed one or more secondary metastatic lesions in four out of five animals, while i.h. infections exhibited metastasis formation in only one out of five animals. Furthermore, in two out of five cases, the primary vesicle implanted into the liver had left the organ and subsequently formed intraperitoneal lesions. The lesions found in mice infected with 100 vesicles varied considerably in size, thus putatively also representing confluent clusters of multiple lesions. Histologically, periparasitic granuloma formation in the liver was observed when a single vesicle was inoculated i.h. (Fig. 2) or when the parasite had metastasized to the liver following i.p. infection. However, the host immune response failed to kill and eliminate a single metacestode and failed to control parasite proliferation subsequent to a lowest-dose infection in all cases.

A second series of experiments tackled the putative role of an intact LL to allow establishment of infection and subsequent metacestode proliferation. In three independent experiments (Table 2, series a, b and c, respectively), mice were infected either with single metacestodes exhibiting an intact LL (Fig. 1a), or with vesicles that had been punctured with a needle but still exhibited basic morphological features of a metacestode, such as a viable germinal layer associated with the outer LL (Fig. 1b, d). Infection successfully progressed in 18 out of 20 mice infected with intact vesicles, thus demonstrating a 90% success in infection when using this minimal infection mode (Table 2). Conversely, when mice were infected with a punctured vesicle, which rendered the viable parasite cells and tissue directly accessible to host effector systems, the infection failed in 19 out of 20 mice. These data indicate that the LL plays a key role in successfully establishing infection. As already shown in the first part of these experiments, the variability in the metacestode weight recovered after necropsy was considerable, although a significant difference could be noted at 4 months p.i. and 7–8 months p.i., respectively.

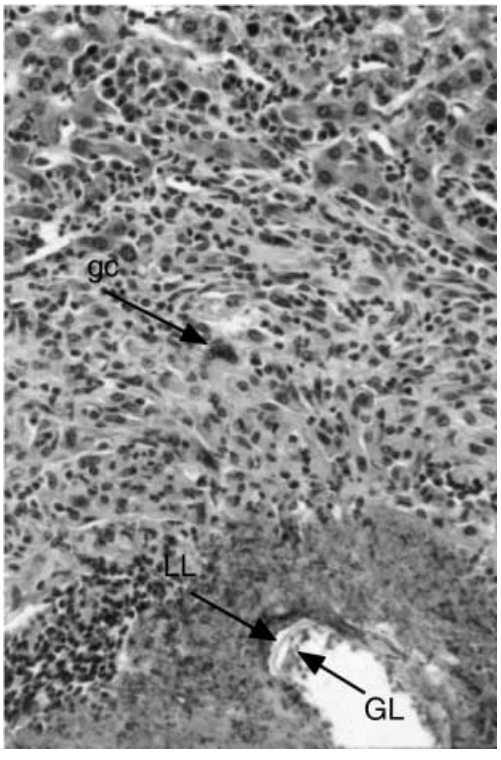

Fig. 2 Liver from a C57BL/6 mouse inoculated intrahepatically with a single metacestode vesicle, shown 20 weeks post-infection (p.i.). Granuloma with giant cell (*gc*) formation surrounding a metacestode vesicle with acellular laminated layer (*LL*) and nucleated germinal layer (*GL*). One representative liver section from five mice with identical lesions is shown. Stain: hemalaun-eosin. *Bar* 100 μm

Assessment of vesicle viability by RT-PCR

Survival of parasite cells following in vitro maintenance was shown in both intact and punctured vesicles by demonstrating qualitatively gene II/3 expression in both samples (Fig. 3). Appropriate RT-PCR products matched in size those obtained with the positive plasmid control (371 bp).

Immune responses

A specific anti-VF antibody response, including all IgG-isotypes, was elicited in all mice infected with intact vesicles (Fig. 4). No significant differences in antibody concentrations were observed between i.p. and i.h. infection modes. Conversely, although inoculation with punctured vesicles induced an IgG1 response in all five infected animals and other IgG isotypes were generated

Table 2 Infection of C57BL/6 mice with a single metacestode vesicle, each exhibiting either an intact or a punctured laminated layer (*LL*), respectively. Mouse numbers marked with either a, b or c indicate three independent but identical experiments, respectively

Mouse number	Vesicle with intact LL PW (mg)	Vesicle with punctured LL PW (mg)	Duration of infection (months)
a1	39	–	4
a2	125	–	4
a3	65	–	4
a4	44	–	4
a5	68	91	4
b1	11,650	–	7
b2	185	–	7
b3	95	–	7
b4	–	–	7
b5	84	–	7
b6	110	–	7
c1	78	–	8
c2	116	–	8
c3	134	–	8
c4	–	–	8
c5	4,120	–	8
c6	13,230	–	8
c7	9,310	–	8
c8	112	–	8
c9	95	–	8

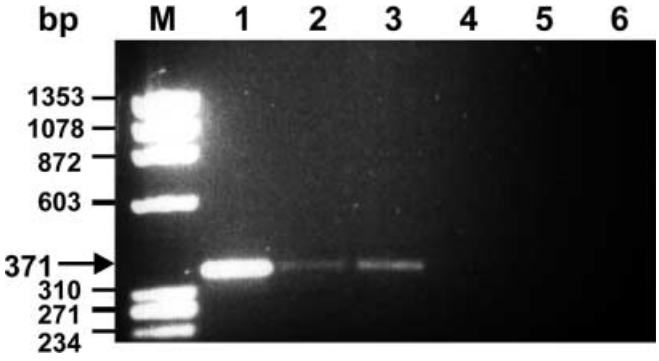

Fig. 3 The gene II/3-specific PCR amplification was performed with DNA from the positive control plasmid pG11 (containing a gene II/3 cDNA sequence; *lane 1*) and cDNA from either intact (*lane 2*) or punctured (*lane 3*) vesicles. A mock control reaction (*lane 4*) and negative control reactions with non-reverse-transcribed total RNA from either intact (*lane 5*) or punctured (*lane 6*) vesicles were also performed. Positions of the 371-base pair (*bp*) PCR product of gene II/3 and size markers (*M*) are indicated at left

in three or four out of five infected animals, the respective mean antibody isotype concentrations were significantly lower for IgG2b and IgG3, when compared with both matching intact-vesicle groups.

Discussion

In this report, we demonstrate that mice infected with *Echinococcus multilocularis* metacestodes fail to control the infection, performed either i.p. or i.h., even when carried out at the lowest possible dose, represented by a single metacestode vesicle. *E. multilocularis* metacestodes are encapsulated by the LL, which represents a microfibrillar matrix composed mainly of high-molecular-weight carbohydrates (Ingold et al. 1999). Based upon several lines of evidence (reviewed by Gottstein and Hemphill 1997), it has been suggested that the LL protects the parasite against host effector mechanisms and thus represents the first line of defense for survival

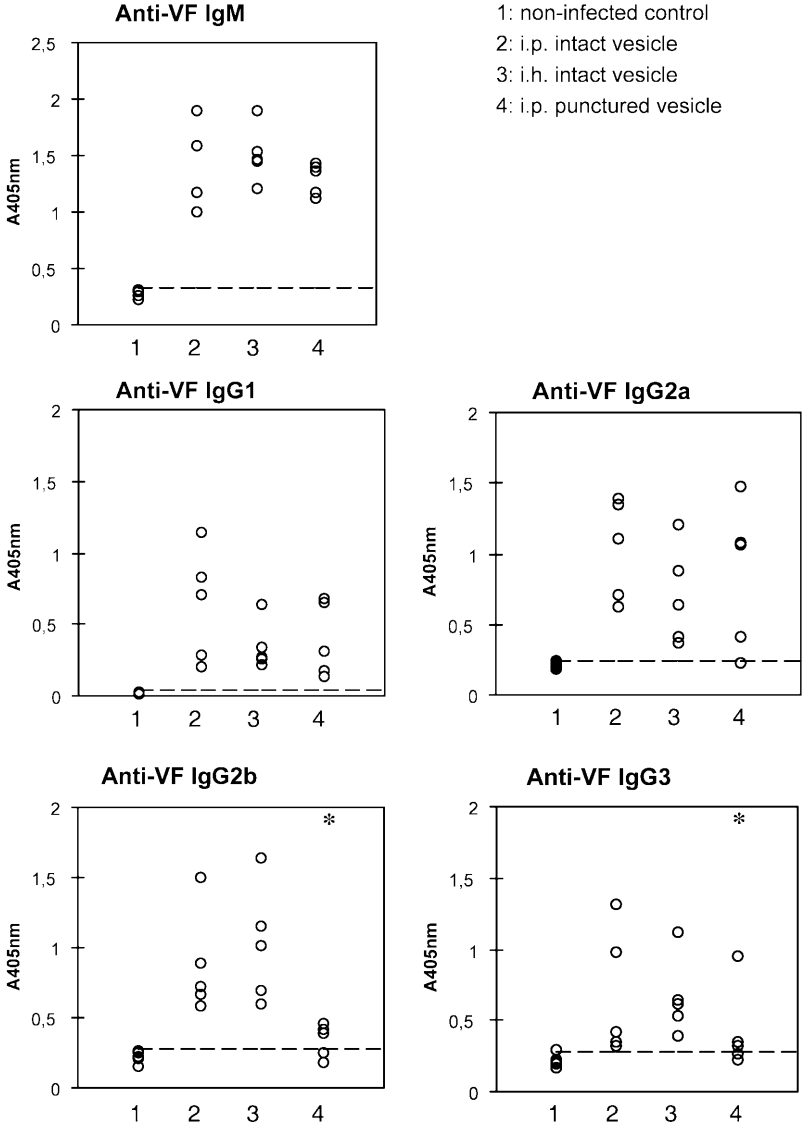

Fig. 4 Specific immune response after infection with a single intact (*2*, *3*) or disrupted (*4*) metacestode vesicle. C57BL/6 mice (five mice/group) were infected intraperitoneally (*i.p.*) or intrahepatically (*i.h.*); and infection was stopped at 4 months p.i.. Specific anti-vesicle fluid (VF) antibody concentrations, including IgM and IgG isotypes, were determined by ELISA using serum of individual control (*1*) or infected (*2*, *3*, *4*) mice, infected using either the i.p. or i.h. route of inoculation. *Each symbol* represents an individual mouse and the *dashed line* indicates the mean plus two standard errors of the negative control group. *Asterisks* indicate statistically significant differences between the marked group and other seroconverting groups. This is one representative out of four independent experiments (with different infection times: 2, 4, 7, 8 months) with, however, identical results

in vivo. This protection has been putatively attributed to the high-molecular-weight carbohydrate content of the LL and its low immunogenicity as a T-cell-independent antigen (Dai et al. 2001; Hülsmeier et al. 2002). Once the metacestode is established, humoral and cellular immune responses in human and murine AE are not sufficient to control parasite proliferation. This is one of the reasons why continuous chemotherapy is required in human patients for nearly unlimited time periods (Ammann and Eckert 1996). In our murine experimental model, infection with a single metacestode vesicle was successfully established in 95% of infected animals, resulting in parasite survival and metastasis formation, despite the induction of specific immune responses. This confirms the hypothesis about the limited effectiveness of the immune responses in controlling parasite survival and growth.

Conversely, once the shield provided by the laminated layer becomes opened, even when only performed by inducing a miniscule hole, the survival potential becomes drastically reduced, in that 95% of such treated vesicles died-out upon experimental infection in mice. So far, we do not know whether death of punctured vesicles is a primarily immune-mediated process (innate or acquired), or only related to non-specific physiological or other biochemical events. The parasite cells and tissue, upon puncture, become exposed to the peritoneal cavity or liver tissue physiology, respectively. From 20 animals infected with punctured vesicles, one still exhibited the presence of a well established metacestode at the end of experiments. Searching for putative explanations, such as incomplete elimination or killing of the germinal layer by the host, we rather concluded that we may have encountered a morphological limitation to obtaining 100% completely punctured vesicles. As shown in Fig. 1c, in vitro-generated vesicles proliferate by exogenous budding, thus forming initially small daughter vesicles, which eventually detach and are released into the medium. Although we carefully selected for uniform vesicles in the first round of experiments (series a1–a5 in Table 2), we may have included – without being aware – a metacestode, such as shown in Fig. 1c, with a small daughter vesicle still attached. In the subsequent experiments (series b1–b6, c1–c9 of Table 2), such metacestodes were carefully avoided and not used for infection.

Still open remains the question as to what mechanisms are responsible for the elimination of punctured vesicles. Claiming that immune-related processes are responsible, we will tackle this hypothesis by performing appropriate experiments in the RAG mouse and other mice with knockouts for various immunological functions. Also, we will introduce in vitro some of these components – such as anti-germinal layer antibodies with or without complement, activated macrophages etc. – to identify specific killing mechanisms challenging the proliferating germinal layer in vitro. Our preliminary in vitro experiments have at least documented that parasite cells survive in vitro maintenance quite well after puncturing. In this respect, viability assessment by RT-PCR proved to be an elegant tool. A similar tool was used to prove the viability of fine-needle biopsies aspirated from liver lesions of AE patients (Kern et al. 1995). However, in vivo survival may become more appropriately documented in the RAG mouse model, as suggested above.

Analyses of the humoral immune response in respective animals showed that the punctured vesicles, following inoculation into the peritoneal cavity of mice, survived a yet undefined time-span. The early phase IgM immune response switched to both Th1- and Th2-associated isotypes IgG1 and IgG2a, similar to what can be observed in mice infected with intact vesicles, thus indicating an active interaction between metabolized and somatic parasite components originating from punctured vesicles. These include proteinic antigens that are predominantly found in VF antigen (Dai et al. 2001). Conversely, IgG3 antibodies, predominantly directed against carbohydrate components, reached only low concentrations in mice infected with punctured vesicles; and values were significantly lower than in mice infected with intact metacestodes. From these findings, we can postulate that, at an early stage of infection, the humoral immune response was relying upon an active host–pathogen interaction. Anti-carbohydrate antigen (predominantly anti-LL) responses appeared delayed in earlier experiments (Dai et al. 2001) and thus would require a long-term parasite survival, a fact which obviously did not occur in the present punctured-vesicle experiments. The inoculated parasite mass (one vesicle) seemed not to be enough to stimulate the respective IgG3 response; and thus we could also claim that the anti-proteinic IgG1/IgG2a response may not have been induced by the inoculated small parasite mass alone, but required interaction between the host and an initially living but subsequently dying-out metacestode. It will be interesting to design appropriate experiments to investigate the time-point of and mechanisms leading to metacestode death.

The findings from our experiments may be linked to those cases of human infection, where disease does not appear. It has been claimed that mass proliferation of the intrahepatic metacestode is putatively inhibited by an appropriate host immune response. The process finally resulted in the early ''dying-out'' of the metacestode, as already shown in several individuals by different authors (Rausch et al. 1987; Bresson-Hadni et al. 1994; Gottstein et al. 2001) in which the lesions had ''aborted''. Lesions are considered to be aborted when they have completely calcified and no viability can be observed after surgical resection of the parasite lesion and subsequent transplantation into susceptible laboratory rodents. Histological and immunological examinations revealed that, in these cases, the parasite material remaining within the lesion consisted largely of debris of the LL (Condon et al. 1988). Surgical removal of the died-out lesion (and thus of the immunostimulatory LL source) resulted immediately in seronegativity with regard to the major antigen of the LL, the anti-Em2 antigen (Lanier et al. 1987). The inability of the patients

to eliminate the Em2-positive LL indicated the very low degradability of this material; and this empirical finding pointed towards the crucial role the LL may play in protecting the parasite from host effector mechanisms.

Conclusively, our results showed for the first time the evidence that infected mice failed to clear infection performed with a minimal, single *E. multilocularis* metacestode unit. The intact LL seems to act as a primary barrier to protect the parasite from host effector mechanisms, since providing access to the inner compartments of vesicles allowed the host to successfully eliminate the metacestode. Our results contribute to a better understanding of the parasite survival strategy and may help to design potential therapeutic tools to treat patients with active AE, by providing better access to the active and obviously vulnerable metacestode germinal tissues.

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