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Echinococcus multilocularis: parasite-specific humoral and cellular immune response subsets in mouse strains susceptible (AKR, C57Bl/6J) or 'resistant' (C57Bl/10) to secondary alveolar echinococcosis

B. GOTTSTEIN, E. WUNDERLIN* & I. TANNER* *Institute of Parasitology, University of Berne, Berne, and *Institute of Parasitology, University of Zürich, Zürich, Switzerland*

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SUMMARY

Parasite-specific humoral and cell-mediated immune responses were investigated in highly susceptible (AKR and C57Bl/6J) and relatively resistant (C57Bl/10) mice undergoing secondary alveolar echinococcosis (infection with *Echinococcus multilocularis* metacestode). The parasite-specific proliferative immune response of lymph node cells upon *in vitro* antigen stimulation remained weak in all three mouse strains. By day 30 p.i., $CD4^+$ lymphoblast cells dominated the total population of blast cells in all three mouse strains. There was, however, an unexpectedly high proportion of $CD8^+$ blast cells; by day 90 p.i., a marked proportional increase in $CD8^+$ cells was seen in susceptible (AKR and C57Bl/6J), but not in resistant (C57Bl/10) mice. Susceptible, but not resistant mice exhibited a significantly decreased responsiveness of lymph node cells to concanavalin A (Con A) stimulation on day 90 p.i. Analysis of the humoral immune response by ELISA showed that resistance in C57Bl/10 mice was associated with the ability of the host to synthesize antibodies to Em2 of the IgG3 and IgG1 isotype. Em2 is a lectin-binding carbohydrate antigen of the laminated layer. In susceptible AKR and C57Bl/6J mice, low levels of anti-Em2 antibodies of the IgG2a isotype were detected. Anti-Em2 antibodies of the IgG3/IgG1 isotype, however, were absent. Differences in subclass-specific IgG responses were confirmed by immunoblot analyses. Our findings suggest that differences in antigen recognition (with respect to subsets of humoral and cellular immune components), probably controlled by non-*H-2* gene(s), coupled to immune suppression modulated by $CD8^+$ cells and/or respective cytokines, may determine susceptibility or resistance in experimental infection with *E. multilocularis*.

Keywords *Echinococcus multilocularis* murine alveolar echinococcosis cellular immunity humoral immunity IgG3 IgG1 Em2

INTRODUCTION

Alveolar echinococcosis (AE), caused by the metacestode stage of *Echinococcus multilocularis*, is one of the most lethal helminthic infections of humans. The natural life cycle involves predominantly rodents as intermediate hosts, where the metacestode parasite develops mainly in the liver as a solid tumour-like and vascularized tissue. Metastasis to various other sites can also occur, such as the peritoneal cavity, the diaphragm, the lungs and the kidney. In human patients and in experimentally infected mice, the growth of the metacestode tissue is always associated with an intense humoral and cell-mediated immune response [1–6]. Generally, humoral and cellular immune reactions, rather than helping the host in controlling parasite proliferation, appear to be involved in immunopathological mechanisms responsible for the occasional chronic granulomatous course of the disease. In some cases, however, the immune system might be able to prevent disease. This is suggested by the finding of patients (upon serological screening by Em2-ELISA) with lesions that have 'aborted' or 'died-out' (lesions are considered to be abortive when no viability can be shown after surgical resection of the parasite lesion and subsequent transplantation of the parasite to susceptible laboratory rodents) [7]. Alaskan patients with a 'died-out' metacestode exhibited high *in vitro* lymphoproliferative responses to *E. multilocularis* crude antigen, in contrast to patients with a still active metacestode [4]. Vuitton *et al.* [8] showed that the periparasitic granuloma, which is mainly composed of macrophages, myofibroblasts and T cells, contained a large number of $CD4^+$ lymphocytes in (French) patients with lesions that had 'died-out'. In patients with active

Correspondence: Professor Dr B. Gottstein, Institute of Parasitology, University of Berne, Längass-Strasse 122, PO Box 8466, CH-3001, Berne, Switzerland.

parasite tissue, on the other hand, the number of $CD8^+$ cells was increased.

Liance *et al.* [5,9] and Bresson-Hadni *et al.* [10] developed murine models which reflect susceptibility (AKR and C57Bl/6) or relative resistance (C57Bl/10) to secondary alveolar echinococcosis. Using these models, they found that a DTH *in vivo* response upon antigen challenge was significantly higher in infected resistant mice than in susceptible mice. A subsequent analysis of the collagen types and of the phenotype of cells within the periparasitic granuloma showed that susceptibility was associated with a persistence of numerous $CD4^+$ lymphocytes and low macrophage number, whereas the periparasitic granuloma of resistant animals showed elevated numbers of $CD8^+$ T cells. Kroeze & Tanner [11] suggested that in mice susceptibility or resistance is probably controlled by non-*H-2* gene(s).

Recent reports showed that $CD8^+$ T suppressor (Ts) cells with a low density of the CD8 antigen ($CD8^{dull}$ cells) were detectable in spleens from mice infected with *E. multilocularis* protoscoleces [12] and in polyclonally activated lymphocytes from normal mice. Further analysis showed that protoscoleces induced $CD8^+$ Ts cells *in vitro* and were assumed to be responsible for immune suppression in mice with alveolar echinococcosis [13]. *Scid* mice reconstituted with purified lymphocytes from infected donor mice were able to suppress metacestode proliferation and protoscolex formation. In contrast, reconstitution with non-immune lymphocytes resulted only in the suppression of metacestode growth [14].

In natural infections, protoscolex formation occurs only in some rodent species and very rarely in human patients. It is widely believed that the laminated layer of the *E. multilocularis* metacestode protects the parasite from host immune reactions [15]. In this regard we have identified an immunodominant antigen of *E. multilocularis* (Em2 antigen) against which we have subsequently generated a MoAb, MAb G11. Immunobiological and immunochemical studies revealed that MAb G11 recognizes a dominant epitope in the laminated layer of the metacestode tissue, which could not be detected in protoscoleces. We obtained experimental evidence that only those *E. multilocularis* metacestode structures which were able to synthesize a laminated layer containing Em2 (as recognized by MAb G11) exhibited the potential to induce secondary alveolar echinococcosis in rodents [15].

The aim of the present study was to analyse potential differences in cellular and humoral events with respect to secondary alveolar echinococcosis in 'resistant' (C57Bl/10) *versus* susceptible (AKR and C57Bl/6J) mouse strains.

MATERIALS AND METHODS

Animals

Three inbred strains of mice, characterized by differing susceptibility to *E. multilocularis* [5,9], were used. The two susceptible strains were AKR (Iffa-Credo, L'Arbresle, France) and C57Bl/6J (Institut für Labortierkunde, University of Zürich, Switzerland); the 'resistant' mouse strain was C57Bl/10 (Institut für Labortierkunde). All animals were raised, housed and handled under conditions corresponding to modern animal experimentation facilities, with free access to germ-free food and water.

E. multilocularis *parasite and antigens*

Crude protein extract antigens were used and prepared as described previously [16], except that a cloned *E. multilocularis* parasite isolate (KF5) was used [15]. The concentration of parasite-specific antibodies in sera was tested by ELISA [17] using both crude *E. multilocularis* extract and the *E. multilocularis* antigen Em2, affinity-purified with solid-phase MoAb MAb G11 [18]. The same MAb G11 affinity-purified antigen Em2 was used for its selective biochemical analysis (see below).

Experimental infection of mice

Three-month-old mice of each strain were injected intraperitoneally (using a syringe and $1{\cdot}2 \times 40$ mm needles) with 50 freshly prepared primary acephalic vesicular cysts suspended in RPMI 1640 [15] to a final volume of 500 μl per animal; control mice received a corresponding volume of RPMI 1640 alone. For serological investigations, groups of five animals per strain were killed, using CO_2, on days 14, 24, 37 and 90 post-infection (p.i.). Sera were stored at -80°C. For cell-mediated immunological testing, groups of five animals per strain were killed on days 0, 30 and 90 p.i. In experiments, where parasite material was sampled (90 days p.i.), metacestode cyst masses were recovered from the peritoneal cavity, and, if already metastasized, carefully dissected from the liver and the peritoneum, making sure that any host tissue was excluded. The metacestode was weighed and investigated histologically for protoscolex formation; viability was assessed by implantation into gerbils [19]. 'Positive' control mice of each strain were immunized by s.c. injection (into the tail root) of 50 μg crude *E. multilocularis* antigen extract emulsified in RIBI adjuvants (total volume per animal 50 μl).

Lymphocyte proliferation assay

Preparation of cells and performance of lymphoproliferative assays were done as described previously [20,21]. Briefly, the inguinal and periaortic lymph nodes were removed aseptically and dissociated into single-cell suspensions by homogenization in a Tenbroek's homogenizer. The cells from all lymph nodes of each animal group, designated with respect to strain and time of collection, were pooled. After washing, the cells were resuspended at appropriate concentrations in tissue culture medium (TCM; RPMI 1640 containing 10% fetal calf serum (FCS), 12 mM HEPES buffer, 50 μM β-mercaptoethanol and supplemented with L-glutamine (2 mM) and penicillin, streptomycin, fungizone (100 U/ml, 100 μg/ml and $0{\cdot}25$ μg/ml, respectively)).

Cells were suspended at a concentration of $0{\cdot}5 \times 10^6$/ml and cultivated at a concentration of $0{\cdot}1 \times 10^6$ cells/well in Nunc 96U tissue culture microplates (Nunc, Roskilde, Denmark; no. 1-63320). All tests were performed in triplicate. Concanavalin A (Con A) stimulation ($2{\cdot}5$ μg Con A per ml) was used as an internal control. Cells were pulsed with 1 μCi/well on the third day of antigen stimulation and harvested on the next day. Results are expressed as Δct/min (mean ct/min of 'positive' probe$-$mean ct/min of control cells without stimulation; a standard deviation of 25% with respect to the mean ct/min was tolerated, data not shown) or as stimulation index (SI; mean ct/min in experimental wells/mean ct/min in control wells).

In parallel to the lymphocyte proliferation assays, larger numbers of identical but non-pulsed cells were used for subsequent immunofluorescence staining and flow cytometry analysis.

Immunofluorescence staining and flow cytometry analysis
Cells proliferating *in vitro* were washed with PBS containing 5% FCS. The cells were stained with PE-labelled anti-L3T4 (GK1.5; Becton Dickinson) and FITC-labelled anti-Lyt-2 (53-6.7; Becton Dickinson) for 30 min on ice. Two-colour flow cytometric analyses were performed using an EPICS Profile Analyzer (Coulter Corp.) equipped with log amplifiers and an argon laser tuned to 488 nm. FITC and PE fluorescence signals were distinguished using 520/30 nm and 575/26 nm band pass filters, respectively, with appropriate compensation. Selective gating of the blast cell population was by the usual procedures according to their typical forward light scatter and right-angled light scatter. Subpopulations identified as being either $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$ or $CD4^-CD8^-$ by immunofluorescence were electronically gated and analysed for their fluorescence signals using linear amplification.

ELISA
Sera were analysed for parasite-specific IgG by ELISA. All serum samples had been kept frozen (−80°C) and were tested at the same time using uniform conditions. The ELISA was performed as described previously [15,22]. Two different *E. multilocularis* antigens were included in ELISA: a crude metacestode protein extract antigen [16] from the *E. multilocularis* parasite clone KF5 [15], and MAb G11 affinity-purified *E. multilocularis* (clone KF5) Em2 [18]. Sera were diluted 1:100; conjugates (all produced in goats and alkaline phosphatase-labelled) used to detect antibody subclasses were anti-mouse IgG1 (Southern Biotechnology Cat. no. 1070-04), anti-mouse IgG2a (Southern Biotechnology Cat. no. 1080-04), anti-mouse IgG2b (Southern Biotechnology Cat. no. 1090-04), anti-mouse IgG3 (Southern Biotechnology Cat. no. 1100-04), anti-mouse IgG (Fc-specific) (Southern Biotechnology Cat. no. 1010-04), anti-mouse IgA (α-chain-specific; Sigma Cat. no. A 7659) and anti-mouse IgM (μ chain-specific; Sigma Cat. no. A 7784).

Immunoblotting
Crude *E. multilocularis* metacestode protein extracts (*E. multilocularis* clone KF5) were resolved by SDS–PAGE and electrophoretically transferred to Immobilon as described earlier [23]. Serum dilutions and conjugates were as described above. Visualization of immunoreactive bands was performed as described by Dao [24].

Preliminary biochemical analysis of affinity-purified Em2 antigen
Echinococcus multilocularis Em2 antigen was purified by affinity chromatography using solid-phase MAb G11 according to the method of Deplazes & Gottstein [18]. Aliquots of 100 µg lyophilized Em2 antigen, each solubilized in 1 ml of PBS, were subjected to the following treatments: (i) boiling for 4 min; (ii) boiling for 4 min at 1 N HCl (immediately neutralized to pH 7·0 after boiling); (iii) boiling for 4 min at 1 N NaOH (immediately neutralized to pH 7·0 after boiling); (iv) protein-digestion [25]; (v) chloroform extraction [25], by testing the water-soluble phase. The influence of the treatments listed above on the antigenic properties of the Em2 antigen was assessed by a solid-phase MAb G11 sandwich-ELISA as described previously [18]. A conventional diagnostic ELISA was also performed, by using the treated Em2 antigen to sensitize directly polystyrene surfaces [26] and testing with a serum from a human patient with proven alveolar echinococcosis (positive control) and a negative human control serum [26].

The DIG glycan detection kit (Boehringer Mannheim, Cat. no. 1142 372) and the lectins and respective inhibitors (Boehringer Mannheim) were used in a dot assay to identify sugars on the Em2 molecule: 1 µg Em2, spotted onto nitrocellulose strips, was tested with the following lectins (carbohydrate specificity in brackets): Con A (α-D-man, α-D-glc); RCA 120 (β-D-gal); SBA (D-galNAc); DBA (α-D-galNAc); UEA I (α-L-fuc); MPA (α-D-gal, α-D-galNAc); PNA (β-D-gal(1-3)-D-galNAc); WGA ((D-glcNAc)2, NeuNAc). Specificity was tested by addition of the respective inhibitors (in brackets): for Con A (D(+)-man); RCA 120 (D(+)-gal); SBA (D-galNAc); DBA (D-galNAc); UEA I (L(-)-fuc); MPA (D-galNAc); PNA (D(+)-gal); WGA (N,N′,N′-triacetylchitotriose). Nitrocellulose strips were incubated for 30 min with the various lectins (in the presence or absence of the corresponding inhibitors) in buffer solutions recommended by the manufacturer. Visualization of reactivity was also according to standard kit procedures.

Statistical analysis
Differences among experimental groups were examined using Student's *t*-test for independent means.

RESULTS

Experimental infection of mice
Susceptibility to *E. multilocularis* metacestode proliferation was primarily assessed by a comparative analysis of the mass of the recovered parasite tissue (Fig. 1). AKR and C57Bl/6J mice harboured large masses of parasite metacestode tissue, and differences between strains were statistically not significant ($P < 0·01$). In contrast, the metacestode burden recovered from C57Bl/10 mice was very low, corresponding only to the volume of the inocula. They were significantly ($P < 0·01$) smaller than those collected from AKR and C57Bl/6J mice. Viability of the metacestodes was maintained till the end of the experiments in all animals of all three strains, as demonstrated by transplanta-

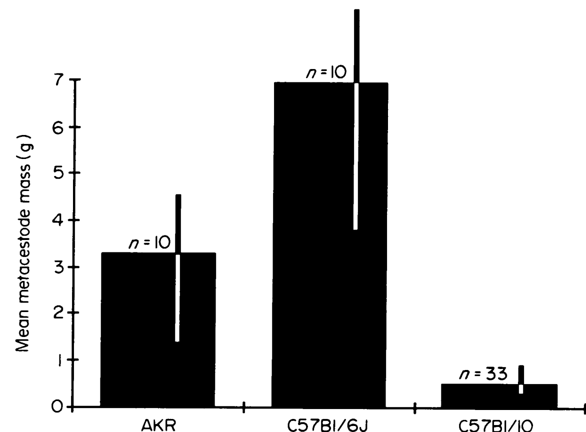

Fig. 1. Mean metacestode tissue mass on day 90 post-infection (p.i.) from susceptible (AKR and C57Bl/6J) and resistant (C57Bl/10) mice. Bars indicate the ranges, and *n* the number of animals per group.

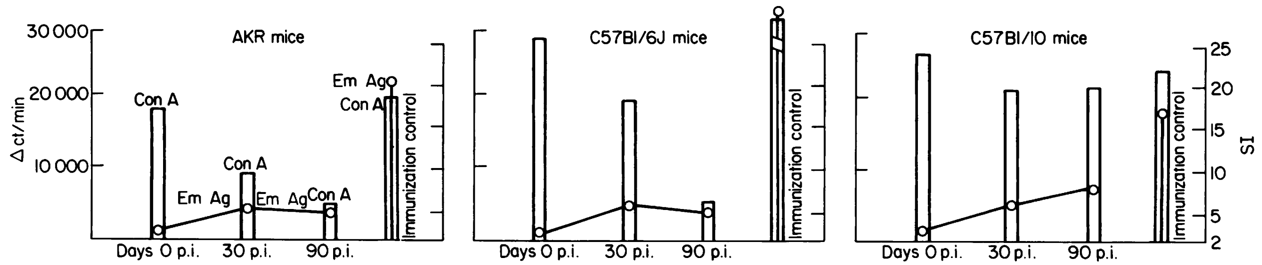

Fig. 2. Proliferative responses of murine lymph node cells reactive to stimulation with *Echinococcus multilocularis* antigen (circles/lines) or Concanavalin A (Con A; histograms) at different times after infection with *E. multilocularis*. Circles represent the parasite-specific stimulation presented as the Δct/min of mean incorporation in five infected animals compared with that in five non-infected control animals. Respective stimulation indices (SI) and a non-infected positive control (mouse immunized with *E. multilocularis* antigen) are both shown on the right.

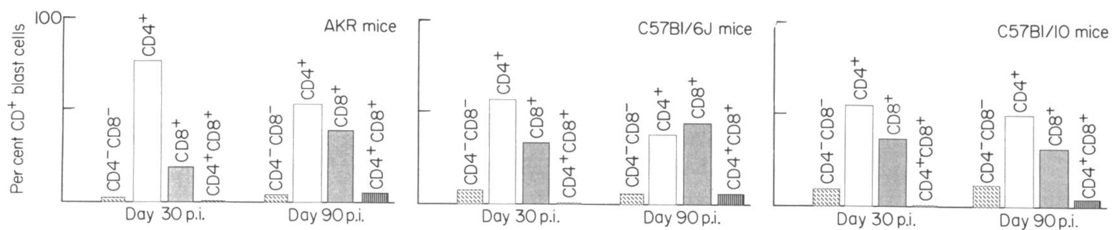

Fig. 3. Two-colour flow cytometric analysis of CD4 and CD8 antigen gating for *in vitro* proliferating blast cell. Stimulation was induced with *Echinococcus multilocularis* crude antigen, and lymphocytes were derived from susceptible (AKR and C57Bl/6J) or 'resistant' (C57Bl/10) mice infected with *E. multilocularis*.

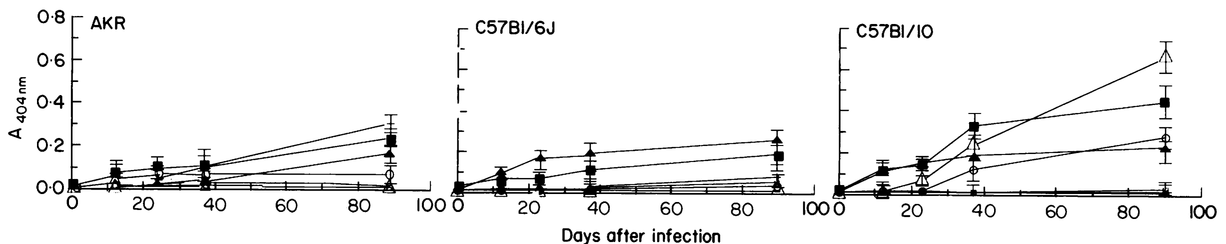

Fig. 4. Comparison by ELISA of the serum concentrations of Em2-specific antibodies of different immunoglobulin classes and subclasses, in susceptible (AKR and C57Bl/6J) and resistant (C57Bl/10) mice, measured at different times after infection with *Echinococcus multilocularis*. Symbols indicate mean values of sera from five mice individually tested, the bars show the range. ○, IgG1; ●, IgG2a; ▽, IgG2b; △, IgG3; ■, IgG; □, IgA; ▲, IgM.

tion into recipient gerbils (data not shown). The presence of protoscolices within metacestode tissues was assessed histologically (periodic acid-Schiff (PAS) stain). Protoscolices were demonstrable in all of 10 AKR mice, in all of 10 C57Bl/6J mice and in three of 33 C57Bl/10 mice killed on day 90 p.i. (data not shown).

Lymphocyte proliferation assay

The lymphocytes obtained from inguinal and periaortic lymph nodes were tested for their proliferative response to *E. multilocularis* crude antigen extract *in vitro* (Fig. 2). Cells from AKR and C57Bl/6J mice showed a relatively low increase in proliferative response on day 30 p.i.; on day 90 p.i. both strains exhibited a decrease in stimulation indices compared with day 30 p.i. In contrast, the SI for C57Bl/10 was increased on day 90 p.i. in comparison with day 30 p.i. The lymphoproliferative response to Con A stimulation was very high on day 0 p.i. for all three strains. A decrease in Con A responsiveness could be shown on day 30 p.i. for AKR and C57Bl/6J mice, the decrease becoming even more evident on day 90 p.i. In contrast, C57Bl/10 mice maintained Con A responsiveness throughout the experiments till day 90 p.i. Immunized control mice all showed a marked response to *E. multilocularis* antigen stimulation and to Con A stimulation *in vitro*.

Immunofluorecence staining and flow cytometry analysis

Figure 3 shows differences among the three different mouse strains relative to the time p.i. in the proportion of $CD4^+$ (helper T cells), $CD8^+$ (suppressor/cytotoxic T cells), $CD4^-CD8^-$ and $CD4^+CD8^+$ lymphocytes. The most striking observation is the relatively high numbers of $CD8^+$ blast cells in all assays, which increased absolutely and also proportionally

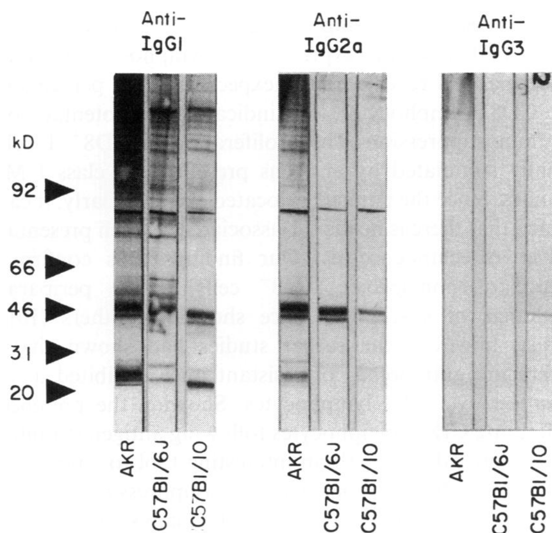

Fig. 5. Immunoblot analysis of IgG1, IgG2a and IgG3 from susceptible (AKR and C57Bl/6J) and resistant (C57Bl/10) mice. All sera were collected from animals on day 90 after infection with *Echinococcus multilocularis*. Molecular weights were estimated with reference to standard molecular markers and are indicated by arrows on the left (mol. wt in kilodaltons).

to the $CD4^+$ blast cells from day 30 p.i. to day 90 p.i. in susceptible mouse strains (AKR and C57Bl/6J). In contrast, resistant mice (C57Bl/10) showed a slight decrease in absolute $CD4^+$ and $CD8^+$ blast cell numbers with an unchanged $CD4^+$/$CD8^+$ ratio. A percentage increase from day 30 p.i. to day 90 p.i. was seen for $CD4^-CD8^-$ and $CD4^+CD8^+$ cells.

ELISA and immunoblotting

Serum immunoglobulin class and subclass concentrations were examined in the three mouse strains at different time intervals after infection with *E. multilocularis*, using crude *E. multilocularis* metacestode extract and the purified antigen Em2. The different mouse strains exhibited marked differences in antibody responsiveness to the Em2 antigen with regard to IgG subclasses (Fig. 4), and the intra-strain variance (depicted by the range) was negligible. The most striking result was the fact that high levels of anti-Em2 antibodies of the IgG3 isotype could be detected in the resistant C57Bl/10 mice which were lacking in the susceptible AKR and C57Bl/6J mice. In addition, C57Bl/10 mice also produced anti-Em IgG1 antibodies in a significant concentration. On the other hand, AKR and C57Bl/6J mice were only able to synthesize anti-Em2 IgG2a at low concentration. Similar serological investigations by ELISA, but using crude *E. multilocularis* metacestode extract antigens, revealed no significant differences between resistant and susceptible animals and the different immunoglobulin subclasses (results not shown).

The reactivity of antibodies belonging to the different IgG subclasses with antigens present in crude *E. multilocularis* metacestode extracts was examined by immunoblot analysis (Fig. 5). All three mouse strains showed very similar qualitative IgG1 banding patterns, but with quantitative differences with regard to some individual bands. Anti-IgG2a immunoblots also revealed no significant differences allowing discrimination between resistant and susceptible mice. A marked IgG3 response to a mol. wt 46 000 polypeptide on the other hand was seen only with serum from resistant mice.

Biochemical analysis of affinity-purified Em2 antigen

A solid-phase MAb G11 sandwich ELISA was used to determine the maintenance of MAb G11 binding capacity after different treatments of affinity-purified Em2 antigen in order to determine some characteristics of this particular molecule. Briefly, the following characteristics (shown in Table 1) were found: boiling of the Em2 antigen for 4 min did not affect antigenicity; boiling for 4 min at 1 N HCl reduced MAb

Table 1. Effects of different treatments of affinity-purified Em2 antigen on antigenicity, measured by MAb G11 binding capacity (assessed by a solid-phase MAb G11 sandwich ELISA) and binding capacity to polyclonal antibodies derived from the serum of a human patient with alveolar echinococcosis (Em2 ELISA)

Treatment*	Mab G11 reactivity before treatment ($A_{405\,nm}$)†	MAb G11 reactivity after treatment ($A_{405\,nm}$)†	Em2 ELISA before treatment ($\Delta A_{405\,nm}$)‡	Em2 ELISA after treatment ($\Delta A_{405\,nm}$)‡
100°C	0·41	0·38	0·68	0·67
100°C, 1 N HCl	0·39	0·15	0·66	0·52
100°C, 1 N NaOH	0·40	0·03	0·63	0·01
Proteinase K digestion	0·36	0·34	0·64	0·64
Chloroform extraction	0·41	0·39	0·66	0·63
Conjugate control§	0·01	0·01	0·02	0·02

* For more detailed information on the treatment procedures, see Materials and Methods.

† Control reactions with an irrelevant solid-phase negative control MoAb of the same isotype were all negative and thus are not shown.

‡ The difference in $A_{405\,nm}$ between the reactivity of the serum from a patient with alveolar echinococcosis and a negative control serum is shown.

§ For the MAb G11 sandwich ELISA, no antigen was included; for the Em2 ELISA, no test serum was included in the test procedure.

Table 2. Lectin-binding characteristics of MAb G11 affinity-purified Em2 antigen in a dot assay with various lectins using the DIG glycan detection kit

Lectin (carbohydrate specificity)	Reactivity with Em2 antigen*
Con A (α-D-man, α-D-glc)	−
RCA 120 (β-D-gal)	+++
SBA (D-galNAc)	+++
DBA (a-D-galNAc)	+++
UEA I (α-L-fuc)	−
MPA (α-D-gal, α-D-galNAc)	(+)
PNA (β-D-gal(1-3)-D-galNAc)	+++
WGA ((D-glcNAc)2, NeuNAc)	+++

* Specificity was tested by addition of respective inhibitors (see Materials and Methods): all reactivities were inhibited by the inhibitors (data not shown).

G11 antibody binding to < 50%, but only marginally affected the binding of polyclonal antibodies from patients; boiling for 4 min at 1 N NaOH destroyed any antibody binding capacity; proteinase K digestion did not affect antigenicity; chloroform extraction did not remove antigenic molecules. In summary, our observations clearly suggest that antibodies bind predominantly to carbohydrate moieties present on the Em2 antigen. Carbohydrate moieties were further characterized by analysing the lectin-binding capacity of MAb G11-affinity-purified Em2 antigen (Table 2). The lectin-binding pattern revealed the presence of the following sugars on the Em2 molecule: β-D-galactose, (α)-D-galNAc, β-D-gal(1-3)-D-galNAc, (D-glcNAc)2, NeuNAc (not taking into account the very weak reaction with MPA).

DISCUSSION

The present study confirms that C57Bl/6J and AKR mice are particularly susceptible to secondary alveolar echinococcosis, whereas C57Bl/10 mice show a relatively high degree of resistance [10]. Susceptibility or resistance in these mice did not appear to be linked to their *H-2* haplotype, since C57Bl/6J and C57Bl/10 animals are both $H\text{-}2^b$. One of the major aims of this study was to search for immunological parameters characterizing either susceptibility or resistance.

Lymphoproliferative responses to *E. multilocularis* crude antigen extract tested 30 and 90 days p.i. were rather weak for all three strains of mice; C57Bl/6J and AKR mice even exhibited a slight decrease in stimulation indices at day 90 compared with day 30. In contrast, the SI for C57Bl/10 increased during this period. A significant difference between resistant and susceptible mice was detected with respect to the lymphoproliferative response to Con A stimulation. AKR and C57Bl/6J mice demonstrated a dramatic decrease in Con A responsiveness at day 30 p.i. and day 90 p.i. In contrast, C57Bl/10 mice maintained their high Con A responsiveness throughout and at the same level as hyperimmunized control mice. The impairment of parasite-specific lymphoproliferative responsiveness during the course of infection thus extended to a general suppression of lymphocyte blastogenesis in susceptible mice, as reflected by a continuous reduction in Con A responsiveness. Analysing antigen-stimulated lymphoblast cells by FACS for the expression of lymphocyte phenotypic surface markers revealed an unexpectedly high percentage of $CD4^-CD8^+$ lymphocytes, thus indicating the potential course of immunosuppression. The proliferation of $CD8^+$ T cells is normally stimulated by antigens presented by class I MHC molecules. Since the parasite is located extracellularly, it can be assumed that there is no class I-associated antigen presentation in alveolar echinococcosis. Our findings thus confirm the presence of non-specific $CD8^+$ cells in the periparasitic granulomas of susceptible mice shown by others [10]. In contrast, however, the earlier studies had shown that the periparasitic granulomas of resistant mice exhibited a lower infiltration by $CD8^+$ lymphocytes. Showing the presence of proliferating $CD8^+$ lymphocytes following antigen stimulation therefore provides us with an interesting tool to study further the potential phenomenon of immunosuppression in alveolar echinococcosis, and we are presently analysing the various cytokines involved to determine potential functional effector markers for resistance.

With regard to humoral immune parameters, we were specially interested in assessing the purified antigen Em2 normally located in the laminated layer [15], as preliminary investigations with crude extract antigen did not result in any subclass-specific sero-reactive discrimination between resistant and susceptible animals. The anti-Em2 immune response in resistant C57Bl/10 mice was marked and associated with IgG3 and IgG1 subclass specificity. Susceptibility of AKR and C57Bl/6J mice on the other hand was associated with a weak anti-Em2 response of the IgG2a isotype, with an anti-Em2-IgG3/IgG1 response completely lacking. Due to its association with resistance, the anti-Em2-IgG3 response in C57Bl/10 mice deserved further consideration. In mice, antibodies directed against polysaccharides are predominantly of the IgG3 isotype [27]. From previous investigations [17,28] we know that the Em2 antigen is not of proteinic nature. The generation of a MoAb (MAb G11; [18]) allowed further characterization of the molecule. The site of expression appeared to be uniquely restricted to the laminated layer of the metacestode, which is known to be rich in carbohydrates, including predominantly galactose, galactosamine and glucosamine [29]. Consequently, we determined in a brief study some preliminary biochemical properties of the Em2 antigen. Proteinase K digestion and chloroform extraction of the Em2 antigen revealed the absence of proteinic or lipid composition. In contrast, lectin-binding tests provided strong evidence for a dominant carbohydrate nature of the Em2 antigen, thus establishing a correlative link to the IgG3 subclass specificity developed by resistant C57Bl/10 mice. In this context it may be worthwhile mentioning the strong anti-Em2 response found in 'resistant' human patients with 'aborted' lesions. Such patients became infected with *E. multilocularis* but appeared to self-cure, and a degenerated and biologically inactive metacestode can subsequently be found [7], thus indicating for the first time the potential of some individuals to kill the parasite by an as yet unknown mechanism. In these patients, the remaining non-viable lesion apparently constantly stimulates the humoral anti-Em2 response [3,30], but ceases to do so upon complete surgical removal of the remaining calcified lesion. Em2 antigen (and consequently metacestode laminated layer) therefore seems to persist in the died-out lesion, independent of the

viability status of the parasite. Preventing the removal of the laminated layer by the host appears to be one of the major tools of the parasite to avoid host immune effector mechanisms, and has already been discussed in previous investigations [15]. The observation that an IgG1 subclass-specific anti-Em2 response exists in resistant mice and an IgG2a subclass-specific anti-Em2 response, albeit weak, in susceptible AKR mice, raises the possibility that the differential induction of IgG subclasses may be regulated by different cytokines, and may thus be Th2- or Th1-restricted. The role of cytokines (IL-4, IL-10 *versus* IL-2, interferon-gamma (IFN-γ)) is presently being investigated.

Differences in subclass-specific IgG responses were supported by immunoblot analyses. Only resistant mice demonstrated IgG3 reactivity against a unique mol. wt 46 000 polypeptide. Although IgG1 and IgG2a analyses provided differences between the three mouse strains, these differences were considered not to be significant enough to discriminate resistance from susceptibility.

In conclusion, this work reveals several interesting features of the anti-*E. multilocularis* response in resistant *versus* susceptible mice. Resistance is accompanied by a predominant anti-Em2 IgG3 response and to a lesser extent by an IgG1 response. IgG3 antibodies also detect a 46 000 polypeptide. All three kinds of antibodies were lacking in susceptible C57BL/6J and AKR mice. This clear difference is presently only correlative, and deserves further investigation with regard to potential causative mechanisms. Susceptibility is accompanied by a marked immunosuppression, as shown by a significant decrease of *in vitro* Con A responsiveness in lymph node cells. In both susceptible and resistant mice, the lymphoproliferative response to *in vitro* stimulation with metacestode antigen remained rather weak. Following antigen-induced mitogenesis, an imbalance of CD4 and CD8 ratios could be observed in proliferating blast cells, which may reflect inhibitory circuits within the T cell compartment.

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