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Cellular immunity in experimental *Echinococcus multilocularis* infection. I. Sequential and comparative study of specific *in vivo* delayed-type hypersensitivity against *E. multilocularis* antigens in resistant and sensitive mice

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SUMMARY

Species- or strain-related differences in receptivity of intermediate hosts to *E. multilocularis* larvae could be related to differences in specific cellular immune response of the host. In order to test this hypothesis, we assessed the delayed-type hypersensitivity (DTH) to *E. multilocularis* antigens (*Em*Ag) in mice of three strains differing by their sensitivity (AKR and C57BL.6) or resistance (C57BL.10) to *E. multilocularis* infection. DTH was determined by measuring *in vivo* the foot-pad response 24 h after an *Em*Ag antigenic challenge. The level of positive response was evaluated in immunized mice; however, a typical DTH response was only observed by immunizing mice with a strong adjuvant schedule. Course of DTH in the immunized mice was shown to be somewhat different in sensitive and resistant mice. The differences were much more marked in mice infected with proliferative *E. multilocularis* larvae. The levels of the footpad response was significantly higher in resistant mice, although the peak of the reaction was obtained later than in sensitive mice. DTH, expressed by the foot-pad response against *Em*Ag, remained significant for the entire period of observation in sensitive as well as in resistant mice. There was no correlation between receptivity of the murine hosts and levels of specific antibodies against *Em*Ag. These results suggest a relationship between resistance to *E. multilocularis* infection and intensity and/or course of DTH in mice. The resistance could be mediated by some particularities of the *in situ* cellular immune response in the periparasitic granuloma.

Keywords *Echinococcus multilocularis* alveolar echinococcosis hydatid disease delayed-type hypersensitivity cellular immunity

INTRODUCTION

Cellular immunity appears as a major mechanism for the control of the growth of *E. multilocularis*, responsible for alveolar echinococcosis. In fact, experimental studies have shown the positive influence of immunosuppression on the development of the larvae (Baron & Tanner, 1976) as well as the negative influence of immunostimulation with BCG on larval growth and metastases (Rau & Tanner, 1975; Reuben, Tanner & Rau, 1978). A depressive effect of the larval growth itself on the specific cellular immune responses of the host has also been suggested (Ali-Khan, 1978). The species- and strain-related differences in sensitivity to *E. multilocularis* infection of intermediate hosts is well documented (Lubinsky, 1964; Lukashenko, 1968), and these differences could be attributed to differences in the immune response of these hosts. However, the comparison of cellular immunity developed in mice of different sensitivities to *E. multilocularis* has not been reported.

The development of a murine model of experimental intrahepatic *E. multilocularis* infection in inbred strains differing in their sensitivity has offered a suitable experimental tool for studying the development of specific immunity during the growth of larvae located in their 'natural,' hepatic environment (Liance *et al.*, 1984). The aim of this study was to assess the course of delayed-type hypersensitivity (DTH) against *E. multilocularis* antigens (*Em*Ag) using a reliable *in vivo* foot-pad response (FPR) test, and to compare the results with the receptivity of three strains of mice to parasitic infection, and with the evolution of the specific humoral immune response. The course of DTH after immunization with an antigenic

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preparation from *E. multilocularis* was also evaluated in each of these strains of mice.

MATERIALS AND METHODS

Animals

Three strains of mice previously identified for their receptivity to *E. multilocularis* (Liance *et al.*, 1984) were used in this study (Iffa-Credo, L'Arbresle, France): two 'sensitive' strains, AKR and C57BL.6 (80% and 90% of positive infections) and one relatively 'resistant strain' C57BL.10 (30% of positive infections) (Liance *et al.*, 1984).

E. multilocularis *antigens*

The antigenic preparation used for immunization or challenge (*Em*Ag) was prepared from parasitic peritoneal lesions maintained in *Meriones unguiculatus*. Jirds were experimentally infected with parasitic cysts obtained from naturally infected *Arvicola terrestris* (Houin *et al.*, 1982). A homogenate was obtained by mincing and freeze–thawing the *E. multilocularis* cysts. The 40000-***g*** pellet fraction obtained from this homogenate was dialysed against distilled water for 1 h at 4°c and then lyophilized (Vuitton *et al.*, 1988).

Immunization of mice

Several schedules were used for immunization of mice with *E. multilocularis* antigenic preparation: (i) s.c. injections of single doses of *Em*Ag (1–6 mg of the lyophilized extract); (ii) multiple injections of low doses of the antigenic preparation (1 mg three to six times); (iii) each of these schedules with Freund's complete adjuvant (FCA); (iv) previous injection (40 μl) of a suspension of BCG K5a (Pasteur Institute, Paris) in saline ($1 \cdot 10^8$/ml), in the right foot-pad of mice, followed 3 weeks later by the immunizing injection of *Em*Ag (40 μl of a solution containing 1·6 mg of *Em*Ag in saline) in the granuloma induced by BCG inoculation, according to Lagrange *et al.* (1978). Results were drawn from five mice for each schedule. Control mice received BCG in the foot-pad without subsequent immunization with *Em*Ag.

Determination of the suitable concentration of Em*Ag to be used for challenge*

The optimum concentration of *Em*Ag to be used for challenge was tested with increasing dilutions of *Em*Ag from 1·2 to 2·4 mg in 40 μl of saline injected in the left foot-pad of mice.

Measurement of the DTH by the foot-pad response

The method described by Lagrange, Mackaness & Miller (1974) was used to assess the DTH against *E. multilocularis* antigens. Measurement of the left foot-pad thickness was performed at 4, 6, 18, 20, 24, 30, 42 and 48 h after challenge in groups of five immunized animals, in order to evaluate the reliability of the test and to determine the optimum time for assessing DTH. Challenge with *Em*Ag and measurement of left foot-pad thickness were subsequently made at the same hour (10 AM) in order to avoid variations due to chronobiology of this immune response.

Infection of mice with E. multilocularis *larvae*

Intrahepatic injection of parasitic larvae maintained in *Meriones unguiculatus* was performed according to the technique previously described (Liance *et al.*, 1984). Control mice received an intrahepatic injection of saline.

Analysis of the course of DTH against E. multilocularis

In groups of five immunized mice of each strain, measurement of the FPR reflecting DTH was made on days 2, 4, 6, 8, 12, 16, 18, 24, 26, 33, 42 and 50 after immunization.

In groups of 10 infected mice of each strain, measurement of the FPR reflecting DTH was made on weeks 1, 2, 3 and 4 and on months 2, 4, 6 and 7 after infection. In the resistant C57BL.10 strain, additional determinations were made at 9 and 10 months after infection.

Measurement of specific antibodies against E. multilocularis

Specific humoral immune response against *E. multilocularis* was evaluated after infection of mice, at the same post-infection times as was specific DTH, using an indirect haemagglutination method adapted to mice, according to Kagan, Norman & Allain (1960). The same antigenic preparation used for the FPR was used for antibody measurements.

Expression of the results

Results of FPR were expressed as the mean foot-pad thickness obtained from the experimental mice in each group ($\pm$s.e.m.), and compared with the mean values obtained from the control mice of the same strain and at the same time post-infection. Statistical analysis of comparisons was made using Student's *t*-test adapted to small samples. Analysis of the course of DTH was made by determining the variance and by using Student's *t*-test for matched samples. The threshold values of significant DTH were determined from results obtained in control mice (mean+2 s.d.), in each strain: 0·13, 0·15 and 0·19 mm in C57BL.10, C57BL.6 and AKR mice, respectively.

Titres of specific antibodies were expressed as 1 to 9 classes. Linear regression analysis was used to test correlations between quantitative values. The value of $P < 0 \cdot 05$ was chosen as level of significance.

RESULTS

Determination of the concentration used for challenge

Concentrations of *Em*Ag lower than 1·2 mg induced transient inflammatory reactions with important interindividual and inter-strain variations; concentrations above 1·76 mg induced very strong long-lasting responses. The concentration of 1·6 mg was thus chosen as challenge dose for all the determinations of FPR.

Course of DTH assessed by the FPR after immunization with Em*Ag*

A significant DTH was never shown after immunization schedules (i), (ii) or (iii), whatever concentration of *Em*Ag, time after immunization and adjuvant we used. An inflammatory response was observed at 4 h in these cases (Fig. 1). A constant and reproducible FPR reflecting a DTH was only demonstrated in mice immunized according to the (iv) schedule using live BCG as adjuvant. No specific DTH to *Em*Ag was observed in mice which received BCG only. Analysis of the kinetics of the specific

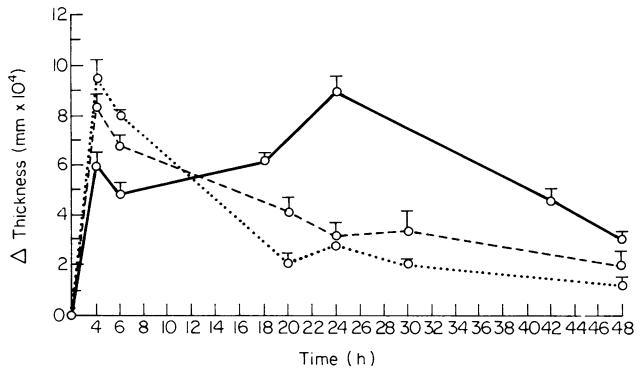

Fig. 1. Dynamics of the foot-pad response (mean ± s.e.m.) obtained 26 days after immunization using *E. multilocularis* antigens (*Em*Ag) without adjuvant (····, 5·6 mg; – – –, 4·8 mg) or after immunization using *Em*Ag (1·6 mg) in a BCG-induced granuloma (,——). The typical course of delayed-type hypersensitivity is observed only with BCG + *Em*Ag (mean values ± s.e.m. after challenge with *Em*Ag, 1·6 mg).

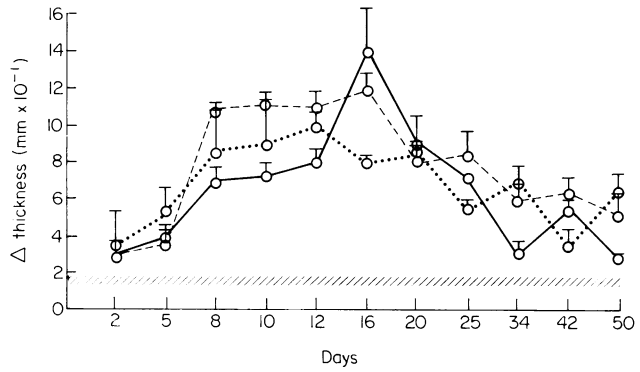

Fig. 2. Course of delayed-type hypersensitivity (mean ± s.e.m.) measured 24 h after challenge with *E. multilocularis* antigens (*Em*Ag) (1·6 mg). The immunization schedule included *Em*Ag + BCG. Results are drawn from five mice in each strain: AKR (– – –); C57BL.6 (····) and C57BL.10 (——). Shaded area indicates the mean + 2SD of the footpad response obtained in control non-immunized mice of each strain.

FPR in immunized mice showed the characteristic course reflecting a DTH with a maximum response obtained at 24 h after challenge (Fig. 1). It was confirmed by histological analysis of the FPR which showed the typical aspect of DTH associating in the mouse, mononuclear and polymorphonuclear cells. The critical time of 24 h was then chosen for all the measurements of DTH using the FPR in our study.

The course of the FPR measured at 24 h after challenge in the three strains of mice following immunization by *Em*Ag according to the (iv) schedule is shown in Fig. 2. The FPR obtained in the immunized mice was significantly higher than that observed in control mice from day 2 after challenge, whatever strain was considered ($P < 0{\cdot}01$). The maximum intensity of the reaction was observed from day 8 and 12 in AKR and C57BL.6 mice, respectively, and on day 16 in C57BL.10 mice. In the C57BL.6 strain, DTH decreased from day 16; it decreased from day 18 in both other strains and a still

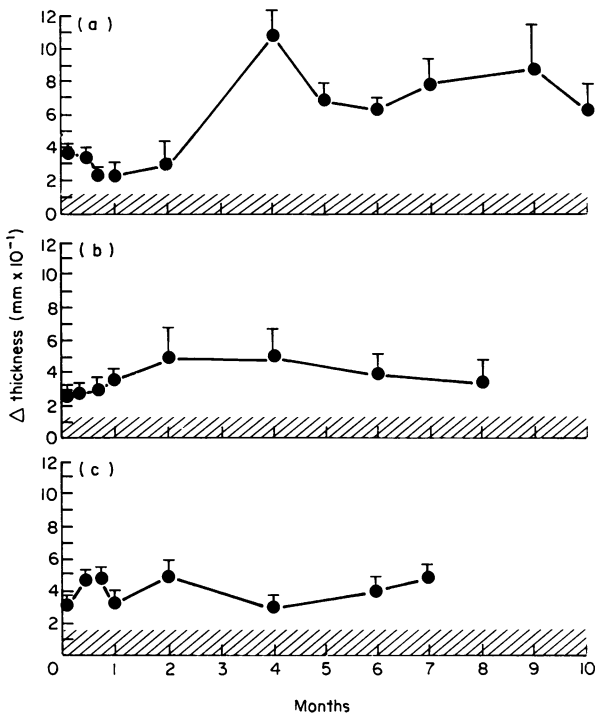

Fig. 3. Course of delayed-type hypersensitivity (mean + s.e.m.) measured after intra-hepatic injection of *E. multilocularis* larvae in the 'resistant' C57BL.10 strain (a) and the 'sensitive' CB57BL.6 (b) and AKR (c) strains of mice. Measurement of the foot-pad response was performed 24 h after challenge with 1·6 mg of *E. multilocularis* antigens (*Em*Ag). Shaded area indicated mean + 2 s.d. of the foot-pad response obtained in control uninfected mice of each strain.

significantly positive plateau was seen up to day 50 ($P < 0{\cdot}01$). A significant difference between the FPR obtained in the three strains was only observed at day 8 (higher in AKR mice) and day 16 (lower in C57BL.6 mice) ($P < 0{\cdot}05$).

Course of DTH assessed by the FPR after infection with E. multilocularis *larvae*

Comparison of the course of DTH against *E. multilocularis* evaluated *in vivo* by the FPR in the three strains of mice infected with *E. multilocularis* larvae is shown on Fig. 3. After the end of week 1, the FPR against *Em*Ag measured at 24 h after challenge was always significantly higher than that observed in control non-infected mice ($P < 0{\cdot}01$). In 'sensitive' AKR mice, values observed at the end of week 2 did not change significantly up to month 2. The peak mean value was obtained at this time. A significant decrease was then observed at month 4 ($P < 0{\cdot}05$), followed by an increase. The FPR remained significantly higher than that observed in control mice, up to the death of the animals due to *E. multilocularis* proliferation ($P < 0{\cdot}01$). The FPR observed in 'sensitive' C57BL.6 mice had the same level of intensity as that observed in the AKR strain. There was a progressive increase up to month 2, followed by a plateau. The course of the FPR observed in the 'resistant' C57BL.10 mice was different. The initial increase of the FPR at the end of week 1 was followed by a significant decrease at week 3 ($P < 0{\cdot}01$). A subsequent major increase was then observed, with a peak obtained at month 4. At that time, and later on, the values

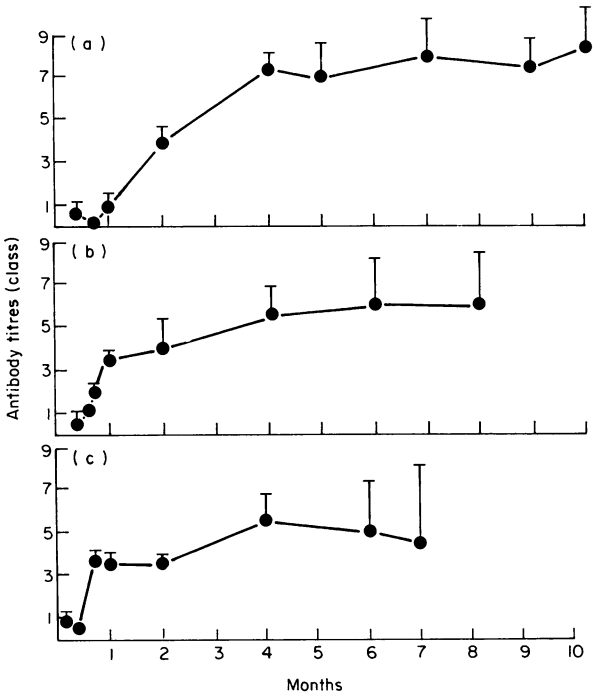

Fig. 4. Course of specific antibody response against *E. multilocularis* antigens (*Em*Ag) after intra-hepatic injection of *E. multilocularis* larvae in the 'resistant' C57BL.10 strain (a) and the 'sensitive' CB57BL.6 (b) and AKR (c) strains of mice. Mean + s.e.m. antibody titres, measured with indirect haemagglutination, expressed as classes 1–9.

observed in C57BL.10 mice were significantly higher than those exhibited by the 'sensitive' C57BL.6 and AKR mice ($P < 0{\cdot}02$). A very important DTH was still observed at months 9 and 10 of the evolution of *E. multilocularis* larvae in C57BL.10 mice. At that time, the foot-pad response was more than five times higher than that of control mice; however, comparison with AKR and C57BL.6 mice was no longer possible, all the mice of these 'sensitive' strains being dead after month 6 of larval growth. Important inter-individual variations were observed in the FPR of the surviving C57BL.10 mice. There was no correlation between individual values of FPR and the size of the parasitic lesions, or the level of specific antibodies in these experimental animals.

Course of the specific humoral immune response after infection with E. multilocularis *larvae*

A progressive increase of the mean level of specific antibodies against *E. multilocularis* antigens was observed in the three strains of mice, up to month 4 after infection (Fig. 4). Strain differences were noted in the first post-infection weeks: at the end of week 3, serum concentrations of antibodies were significantly higher in AKR mice than in C57BL.10 mice ($P < 0{\cdot}05$); the mean levels of antibodies observed in C57BL.6 mice were intermediate, but not different from those observed in both other strains. Levels of antibodies plateaued up to the end of the observation time in the three strains; the average serum concentrations of antibodies measured in C57BL.10 mice were higher than those measured in the 'sensitive' mice; however, important inter-individual variations were observed and the difference was not significant. There was no correlation between the levels of specific antibodies and those of DTH in any strain.

DISCUSSION

The results obtained in this study of the cellular immune response against *E. multilocularis* showed that a significant specific DTH assessed *in vivo* using the FPR was observed in strains of mice known for their relative sensitivity or resistance to *E. multilocularis* infection. However, marked differences were noted in the intensity and course of the DTH in resistant mice compared with sensitive mice; they suggest that resistance to *E. multilocularis* infection could be mediated by the cellular immune response of the host, while antibody production is not clearly associated with the receptivity of intermediate hosts.

Study of DTH *in vivo* using the FPR has been shown to be a useful and reliable tool for evaluating the importance and/or course of cellular immune response in experimental models of infectious diseases, after immunization or infection (Hurtrel & Lagrange, 1978; Lagrange *et al.*, 1978, 1983; Louis *et al.*, 1984). However, accurate determination of the various parameters involved in this method is a prerequisite for the reliability of the test. In preliminary experiments, we used the schedule of immunization and challenge described by Ali-Khan (1978) and we did not obtain any reliable DTH but only non-specific inflammatory responses. We therefore decided to control every parameter of the reaction using mice immunized with *E. multilocularis* extracts in order to assess the level of 'positive' DTH responses. Analysis of the results obtained with different schedules of immunization showed that antibody secretion could be induced by almost every schedule, and particularly by *Em*Ag associated with FCA. In contrast, only the schedule involving a previous induction of granuloma by BCG was able to induce a significant cellular immune response, revealed by the FPR. It is well known that many antigens from micro-organisms are unable to induce a cellular immune response *in vivo* when they are used without FCA in order to immunize animals (Hurtrel & Lagrange, 1978). We showed in a preliminary study that *Em*Ag + FCA was able to sensitize mice, when tested using the specific proliferative response of lymphocytes from the draining lymph node (Vuitton, 1985). However, a stronger adjuvant effect was required for *in vivo* expression of the response. This requirement has already been described with other micro-organisms, and the modifying effect of BCG on the induction of T cell response is now well documented (Mackaness, Lagrange & Ishibashi, 1974; Lagrange *et al.*, 1978). Our observations in immunizing mice could be considered as the counterpart of those made by Rau & Tanner (1973) on the protective effect of BCG in mice infected with *E. multilocularis* larvae.

In fact, our results obtained both in immunized and infected mice support the hypothesis of strain-related differences in cellular immune response, capable of modulating the larval growth of *E. multilocularis*. Comparison of the course of DTH obtained by immunizing mice with *Em*Ag with that observed after immunization with BCG and challenge with tuberculin extracts (Lagrange *et al.*, 1983) shows that maximum intensity of FPR obtained with *Em*Ag appeared earlier and was twice as high as that obtained with tuberculin. It was particularly high in the resistant strain and peaked somewhat later than in sensitive

strains. It decreased from the week 3 after immunization, as is usual with protein antigens but remained significantly positive up to 8 weeks. All these observations suggest that a cellular immune response was difficult to reveal *in vivo* after immunization with *Em*Ag and required a strong adjuvant procedure; but once induced, it was very important, long-lasting and dependent on the strain of mice used for immunization. Inter-strain differences were more marked after infection of mice with *E. multilocularis* larvae. In resistant strains, the development of specific DTH was slower, the peak was higher, as was the subsequent plateau, compared with sensitive strains. In long-living animals of the C57BL.10 strain, DTH never became negative even at month 10 of the larval growth. These results are in opposition to those obtained by Ali-Khan (1978) in C57BL.6 mice. He observed negative reactions at week 12 post-infection. In the three strains of our study, a decrease was observed at the end of week 2 in C57BL.10 and at month 4 in AKR mice. However, the FPR remained consistently higher than that measured in control mice. Our results are in keeping with observations made in human alveolar echinococcosis (Vuitton, 1985): cellular immune response against *Em*Ag assessed *in vitro* by testing proliferation of circulating mononuclear cells has remained significantly positive for years after the diagnosis of alveolar echinococcosis. Results reported by Ali-Khan (1978) could be due to technical reasons; we never obtained a significant DTH response by using his technique, and analysis of his results shows that the FPR were higher at 6 h than at 24 h, reflecting perhaps a non-specific inflammatory response.

No direct correlation between the development of the parasitic lesions in an individual animal and the value of the FPR and/or titre of specific antibodies was obtained in our study. However, the association of a DTH of stronger intensity and of different kinetic aspect with the resistance of the C57BL.10 strain, assessed by the low percentage of positively infected animals and by the long survival period of these infected animals, suggest that resistance of a strain is related to the development of cellular immune response. Qualitative or quantitative differences in the periparasitic granuloma have been reported in patients with abortive alveolar echinococcosis, compared with patients with evolutive disease (Vuitton *et al.*, 1989). The relationship between the phenotypic aspect of the granuloma and DTH in the three strains under study is reported in an accompanying paper (Bresson-Hadni *et al.*, 1990). It suggests strongly that genetic influences on the receptivity to *E. multilocularis* infection could be mediated by quantitative differences in DTH as well as proliferative differences in the cells which are recruited locally to control the development of the parasitic larvae.

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