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Idiotypic modulation of the antibody response of mice to *Echinococcus granulosus* antigens

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SUMMARY

In this work we analysed the modulation of the antibody response to *Echinococcus granulosus* antigens via anti-idiotype (Ab2) administration. In a first set of experiments, we determined the antibody response to hydatid cyst fluid antigen (HCFA) of mice immunized with Ab2. Results showed that Ab2 elicited anti-HCFA antibodies in mice that had never been exposed to HCFA before. Moreover, that response was characterized by booster effect, avidity maturation and an inverse correlation between avidity and Ab2 dose. We infected these mice and measured the titre and avidity of anti-HCFA antibodies during 250 days of infection. Lack of avidity maturation was the most important feature observed, suggesting that the parasite (either protoscolex or cyst) could modulate the antibody response of its host. In a second set of experiments, we investigated the presence of regulatory anti-idiotypes in the Ab2 preparation. In this context we treated neonates with different doses of Ab2, immunized them with HCFA 12 weeks later and determined their anti-HCFA titres. A specific and Ab2 dose-dependent suppression of the response to HCFA was observed, suggesting the existence of regulatory anti-idiotypes in the Ab2 preparation. Although this Ab2 could be involved in the regulation of the anti-HCFA response, other anti-idiotypes could also be involved. Our results show that it may be possible to improve the avidity of the anti-HCFA response via the administration of the anti-idiotype Ab. However, the live parasite could successfully revert this effect by mechanisms not yet characterized.

INTRODUCTION

Hydatid disease produced by the tapeworm *Echinococcus granulosus*, affecting both humans and domestic animals, is one of the most important zoonoses in the world.[1] In endemic areas infection with the metacestode is a major public health problem and a cause of important economical losses.[2–4]

Protective immunization against hydatid infection, using *E. granulosus* oncospheres, is feasible according to Heath *et al.*[5] None the less, the high pathogenicity of oncospheres severely limits their use for vaccines. The use of anti-idiotypic antibodies for vaccination has been proposed by several authors.[6–12] Idiotype vaccines could be useful in the case of parasitic diseases in which the parasite itself cannot be used as immunogen and appropriate amounts of purified protective antigens (i.e. glycoproteins) are not available. Strong protection to *Trypanosoma brucei rhodesiense* and to *Schistosoma mansoni* has been achieved using anti-idiotypic antibodies.[9,13] Some possible drawbacks of the use of anti-idiotypic vaccines are their genetic restriction and their disregulatory action on the immune system. We have previously demonstrated the lack of genetic restriction, but so far it is not clear whether this kind of immunogen affects the host immune response to *E. granulosus*.[14]

In this work we analysed whether a previous immunization with anti-idiotypic antibodies (Ab2) can modulate the antibody response against live parasites. For that purpose, we compared the antibody response during infection of mice immunized with Ab2 or with hydatid cyst fluid antigen (HCFA). In addition, we analysed the presence of regulatory anti-idiotypes in the Ab2 preparation in HCFA-immunized mice that had been neonatally treated with Ab2.

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Abbreviations: Ab, antibody; Ab1, idiotype; Ab2, anti-idiotype; HCFA, hydatid cyst fluid antigen; PSC, protoscolex.

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MATERIALS AND METHODS

Mice

CD1 mice, originally obtained from Charles River Breeding Laboratories Inc. (Wilmington, MA) and subsequently bred at the Facultad de Química, Montevideo, Uruguay, were used for infections and immunizations.

Hydatid cyst fluid

HCFA was obtained according to Carol *et al.*[14] from bovine

hydatid cysts. Briefly hydatid cysts were asceptically punctured and cyst fluid (containing protoscolices and membrane fragments) aspirated. Protoscolices (PSC) were allowed to decant for 20 min at room temperature and the fluid was carefully removed. Immediately after sedimentation, 0·02% sodium azide (preservative) and 5 mM EDTA were added to avoid proteolysis.[15] Finally fluid was extensively dialysed against distilled water and freeze-dried.

Protoscoleces

PSC, obtained as described, were washed with phosphate-buffered saline (PBS) to separate non-viable PSC and membrane debris. Viability was evaluated by 5% eosin exclusion; PSC exhibiting more than 95% viability were used.

Human hHV-Id (Ab1)

Serum from a hydatid-infected patient was selected for idiotype preparation (hHV-Id). This serum exhibited a high anti-hydatid antibody titre by enzyme-linked immunosorbent assay (ELISA), and an immunoelectrophoretic pattern that revealed a broad antigen specificity repertoire related to a reference serum (Pan American Zoonosis Center/WHO, Buenos Aires, Argentina).[16] Specific antibodies were affinity purified with HCFA.

Rabbit anti-hHV-Id (Ab2)

One rabbit was immunized with hHV-Id according to Carol *et al.*[14] Human immunoglobulins coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) were used to adsorb non-anti-idiotypic antibodies from rabbit serum. Rabbit immunoglobulins coupled to the same matrix were used to adsorb remaining rheumatoid factor-like activity from the same serum. The adsorbed serum retained its reactivity against hHV-Id but not against normal human immunoglobulins, as assayed by ELISA using normal rabbit serum as control.

Rabbit anti-anti-hHV-Id (Ab3)

Rabbit Ab3 serum was prepared by Carol *et al.*[14] The same rabbit from which Ab2 was obtained was stimulated with its own anti-Id antibodies. After bleeding, immunoglobulins were precipitated by addition of saturated ammonium sulphate solution to 50%, and extensively dialysed against PBS. These antibodies recognized a broad spectrum of antigen fraction similar to that recognized by serum of a rabbit immunized with HCFA.

Neonatal Ab2 treatment

Groups of five mice were treated intraperitoneally (i.p.), within the first day of life with 0, 5, 100 or 150 ng Ab2 per mouse. Twelve weeks later mice were primed i.p. with 50 μg HCFA in complete Freund's adjuvant (CFA; 1 : 1, v/v). On week 15 they were boosted i.p. with the same dose in incomplete Freund's adjuvant (IFA; 1 : 1, v/v). Mice were periodically bled up to day 220 post-priming and sera stored at −20°.

Immunization with HCFA

Five adult CD1 mice were immunized i.p. with 50 μg of HCFA in CFA (1 : 1, v/v) on day 0 and with 50 μg HCFA in IFA (1 : 1, v/v) on day 27. Mice were periodically bled and sera stored at −20°.

Immunization with Ab2

Three groups of five adult CD1 mice were immunized i.p. with either 5, 10 or 50 μg of Ab2 or 50 μg of HCFA in CFA (1 : 1, v/v) on day 0; all were boosted i.p. with 10 μg of Ab2 in IFA (1 : 1, v/v) on day 27. Mice were periodically bled and sera stored at −20°.

Infection

Mice primed with 5 or 10 μg of Ab2 were infected i.p. 6 months later with 2000 viable PSC per mouse according to Araj *et al.*[17] Five non-immunized mice were infected in the same way and used as controls. Mice were periodically bled during 8 months, and sera stored at −20°.

Determination of anti-HCFA antibodies

Antibody titre was determined by ELISA according to Carol *et al.*[14] Briefly, 96-well microtitre plates (Nunc, Roskilde, Denmark) were coated with 50 μg/ml HCFA.[18] Serial dilutions of the samples were incubated for 3 h at room temperature. As second antibody, peroxidase-conjugated sheep anti-mouse immunoglobulins (Sigma, St Louis, MO) were added and incubated overnight at 4°. The substrates, 3-methyl-2-benzothiazolinone hydrazone (MBTH; Sigma) and 2-dimethylaminobenzoic acid (DMAB; Sigma) were used.[19] The enzymatic reaction was stopped with 2 M sulphuric acid, and optical density at 600 nm (OD 600) measured (Titertek Multiskan Plus reader; Flow Laboratories, Helsinki, Finland). A pool of sera from experimentally infected mice was titrated by diffusion in gel–ELISA (DIG–ELISA) and used as reference serum.[20] Data (OD 600) corresponding to each sample were converted to the antibody concentration equivalent to this reference for analytical consistency.[21]

Determination of the avidity index

Avidity indices of anti-HCFA antibodies were determined according to Pullen *et al.*[22] Briefly, microtitre plates were coated with HCFA, blocked with bovine serum albumin (BSA) and washed as described above. Each serum sample diluted in PBS–Tween–BSA was dispensed in eight wells (50 μl/well) and incubated for 3 hr at room temperature. Then 50 μl/well of 0, 0·1, 0·2, 0·4, 0·8, 1·6, 3·2 and 6·4 M potassium thiocyanate (KSCN) in PBS–Tween–BSA was added and the plates incubated at room temperature for 15 min prior to washing and addition of the conjugate. The rest of ELISA protocol was the standard one. Antibody concentrations equivalent to the reference were calculated as described above. The concentrations in the absence of KSCN were assumed to represent effective total binding of specific antibody; antibody concentrations in the presence of increasing concentrations of KSCN were converted to the percentage of the total bound antibody. Linear regression analysis of the $\log_{10}$ (percentage binding) versus molar concentration of KSCN were carried out, and the avidity index representing the molar concentration of KSCN required to reduce the initial antibody concentration to 50% was calculated.

Different determinations for the same sample gave a coefficient of variation of 14.

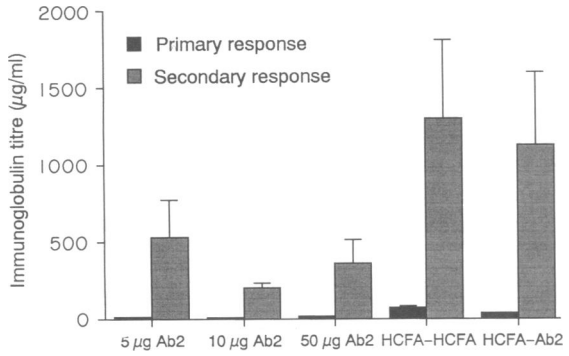

Figure 1. Anti-HCFA antibody titres in primary and secondary responses. Four groups of five adult mice were primed with either 5, 10 or 50 μg Ab2 or 50 μg HCFA and all boosted with 10 μg Ab2 on day 27. A fifth group of five adult mice was primed and boosted with 50 μg HCFA on day 0 and 27, respectively. Anti-HCFA antibody titres were determined by ELISA for primary and secondary responses (day 14 and day 60, respectively). Results represent the mean values ± SD.

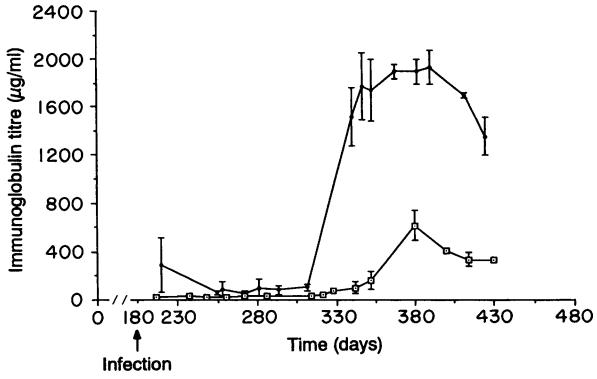

Figure 3. Anti-HCFA antibody titre during infection. Five adult mice were primed with 5 μg Ab2 and boosted (day 27) with 10 μg Ab2 (●); 6 months later they were infected i.p. with 2000 PSC (day 180). A second group of five adult non-immunized mice were infected and used as control (□). All mice were bled periodically, and anti-HCFA antibody titres were determined by ELISA. Results represent the mean values ± SD.

RESULTS

Antibody responses of mice immunized with HCFA and/or Ab2

Figure 1 shows that Ab2-immunized mice produced anti-HCFA-specific antibodies and their concentrations were higher in the secondary than in the primary response, regardless of the Ab2 dose used for immunization. In the primary response an inverse correlation was observed between anti-HCFA Ab avidity and Ab2 dose, as could be expected (Fig. 2). However, that was not observed in the secondary response; similar avidity indices were observed in sera from mice immunized with either 5 or 10 μg of Ab2. Avidity maturation was observed in the case of mice immunized with 10 μg of Ab2 and with 50 μg of HCFA, even though the former exhibited greater avidity indices than the latter. The antibody titre and avidity index of HCFA-primed and Ab2-boosted animals were similar to those of mice primed and boosted with HCFA.

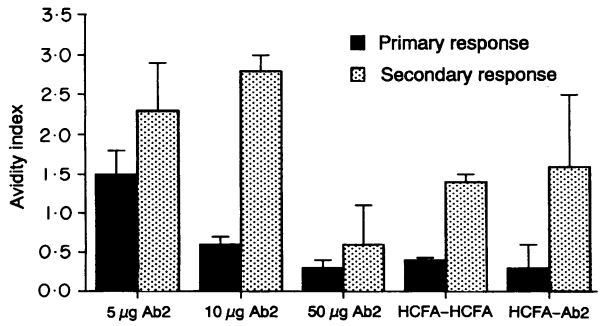

Figure 2. Anti-HCFA antibody avidity index in primary and secondary responses. Four groups of five adult mice were primed with either 5, 10 or 50 μg Ab2 or 50 μg HCFA and all boosted with 10 μg Ab2 on day 27. A fifth group of five adult mice were primed and boosted with 50 μg HCFA on day 0 and 27, respectively. Avidity indices were determined by ELISA for primary and secondary responses (day 14 and day 60, respectively). Results represent the mean values ± SD.

Antibody response during infection of Ab2-immunized mice

In order to analyse if previous Ab2 immunization could modulate the antibody response to live parasites, mice that had been immunized with 5 or 10 μg of Ab2 were infected and the titres and avidity indices of their anti-HCFA antibodies were compared with the corresponding values from non-immunized mice (control group). Figure 3 shows the anti-HCFA antibody titres during infection of mice immunized with 5 μg of Ab2 and of non-immunized mice. Although the profiles corresponding to both groups were similar, the titres of Ab2-immunized mice were higher than those of non-immunized mice. Mice immunized with 10 μg of Ab2 showed titres similar to those of mice immunized with 5 μg of Ab2 (data not shown). Figure 4 shows the avidity indices of anti-HCFA Ab from immunized and control mice. Avidity maturation was not observed in any group. Moreover, although mice immunized with 10 μg of Ab2 showed higher avidity than the other groups up to day 310, the avidity index decreased to values similar to those of the control

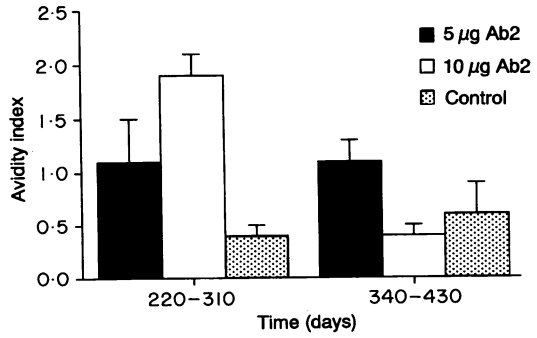

Figure 4. Avidity indexes of anti-HCFA antibodies during infection. Two groups of five adult mice were primed with either 5 or 10 μg Ab2, and all boosted with 10 μg Ab2 on day 27. Six months later they were infected i.p. with 2000 PSC (day 180). A third group of five adult non-immunized mice were infected and use as a control. All mice were bled periodically, and anti-HCFA antibody avidity indices were determined by ELISA. Results represent the mean values ± SD between days 220–310 and days 340–430 post-infection for each group.

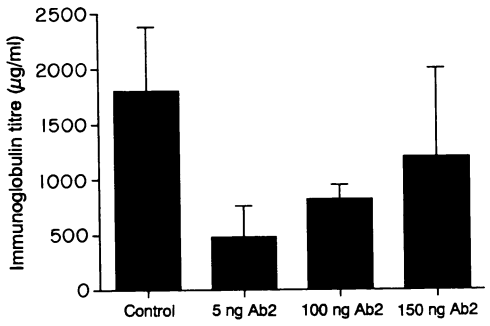

Figure 5. Anti-HCFA antibody titres in Ab2-treated neonates. Three groups of five neonate mice were treated i.p. with 0, 5, 100 or 150 ng Ab2. Twelve weeks later mice were primed i.p. with 50 μg HCFA and boosted with the same dose in week 15. Mice were periodically bled until day 220 post-priming, and the anti-HCFA antibody response analysed by ELISA. Results represent the mean values ± SD of maxima anti-HCFA titres for each group.

group after day 340. It should be noted that a minimum avidity index was observed when the anti-HCFA Ab titre reached its maximum level (day 340).

Suppression of the anti-HCFA response by neonatal treatment with Ab2

We analysed the presence of regulatory anti-idiotypes in the Ab2 preparation further. This was performed by Ab2 administration to neonates, HCFA immunization 12 weeks later and analyses of the corresponding anti-HCFA antibody response. Results in Fig. 5 show that a suppression of the anti-HCFA response Ab was observed in adult mice that had been neonatally treated with Ab2 doses of 5 or 100 ng, while this suppression was not apparent in neonates treated with a higher Ab2 dose (150 ng).

DISCUSSION

In this work we analysed the modulation of the antibody response to *E. granulosus* antigens via Ab2 administration. In the first set of experiments, the anti-HCFA antibody response of mice immunized with either Ab2 or HCFA was compared, in order to test the presence of internal image in the polyclonal Ab2 and to evaluate its ability to mimic hydatid antigen. Results shown in Fig. 1 indicate that Ab2 evoked an anti-HCFA antibody response similar to that elicited by HCFA itself, since Ab2 as able to stimulate the production of specific anti-HCFA ntibodies in animals that had never been exposed to HCFA before. Furthermore, booster effect, avidity maturation and an inverse correlation between anti-HCFA Ab avidity and Ab2 dose were observed in all Ab2-immunized mice, as well as in those immunized with HCFA (Fig. 2). These characteristics are consistent with those of the antibody response to conventional T-dependent antigens. In addition, HCFA-primed mice mounted a specific anti-HCFA secondary response after being boosted with Ab2, similar to those primed and boosted with HCFA (Figs 1 and 2). From the above results, we expected mice primed with 5 and 10 μg of Ab2 to make more evident, during infection, the effect of a previous immunization with Ab2. In fact, these mice presented higher avidity indices than other groups of animals, including HCFA-primed mice. Therefore, mice primed with 5 or 10 μg of Ab2 were infected to carry out the second set of experiments, i.e. the study of the Ab response during infection of Ab2-immunized mice. We compared the Ab response during infection of those mice previously primed with 5 or 10 μg of Ab2, with that of a third group of untreated mice. The difference in titre of the Ab2-primed mice with respect to control mice (Fig. 3) could be due to the stimulation of the HCFA-specific memory cells generated in the immunized animals by internal image Ab2 antibodies mimicking HCFA. During infection, Ab2-immunized mice had higher anti-HCFA titres than controls, but avidity maturation was not observed in any group (Fig. 4) even though it was observed during the previous immunization (Fig. 2). These results suggest that the live parasite could modulate the antibody response of its host. The high titres of specific antibodies, together with the lack of avidity maturation, could be due to T-independent antigens and/or polyclonal activators produced by the parasite.[23,24] Further experiments, such as *in vitro* stimulation of lymphocytes with purified parasite antigens or *in vivo* analysis of the T-dependence of parasitic immunogens, should be performed in order to test the above hypothesis.

It should be mentioned that with the infection protocol used, *E. granulosus* PSC develop to cysts in approximately 3 months (G. Ferragut, personal communication). This means that up to that moment the immune system of infected mice will not recognize HCFA epitopes unless they cross-react with molecules expressed by the differentiating PSC. This could account for the low anti-HCFA titres observed in all infected mice up to day 310 (day 130 post-infection), when it was expected that cysts had recently been established in the host (Fig. 3).

Another interesting result was the decrease in avidity index observed during infection in mice primed with 10 μg of Ab2. This decrease would support the above mentioned hypothesis, but it is not consistent with the high avidity apparently associated with some idiotopes recognized by Ab2, suggested by the results in Fig. 2. A possible explanation may be that Ab2 also contains Ab to regulatory idiotopes that are somewhat influenced by the live parasite, thus affecting in a different way the Ab responses during immunization and infection.

In addition, the Ab2 dose-dependent anti-HCFA antibody suppression induced by neonatal treatment with Ab2 (Fig. 5) further support the existence of regulatory anti-idiotypes in Ab2. This is consistent with the fact that exposure of neonates to anti-idiotypic antibodies influences the selection of available repertoires.[25] Taken collectively, our results suggest that Ab2 can mimic *E. granulosus* antigens, but it induces an antibody response characterized by lower titres and higher avidities than that elicited by parasitic antigens. Furthermore, Ab2-immunized mice, once infected, showed higher anti-HCFA Ab titres than control mice. Anti-HCFA Ab avidity was also higher than that of control mice, but only until day 310, suggesting the involvement of some kind of polyclonal activator and/or T-independent antigens. These facts suggest that manipulation of the anti-HCFA antibody response to improve avidity via stimulation of silent idiotypes may be possible. None the less, the parasite could revert this effect by other mechanisms. This should be analysed together with the involvement of different T-cell populations in the observed antibody response.

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