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Antibody response of *Echinococcus granulosus* infected mice: Protoscolex specific response during infection is associated with decreasing specific IgG1/IgG3 ratio as well as decreasing avidity

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SUMMARY

The antibody response was followed during 68 weeks in 17 Balb/c mice intraperitoneally (i.p.) infected with Echinococcus granulosus *protoscoleces (PSC) and in three mice i.p. immunized with dead PSC. Titres of antibodies recognizing peptidic and glucidic PSC epitopes, as well as their isotypic and avidity profiles were followed by ELISA. In addition, antigen recognition patterns were analysed by immunoblot. The response against carbohydrate epitopes was dominant in infected and immunized mice but stronger in the first group. Infected mice showed similar profiles of specific IgG and IgM with maximum titres from week 38 to 53. Although IgG1 and IgG3 were the predominant antibody subclasses, the ratio of IgG1/IgG3 antibody titres as well as antibody avidity decreased during the experiment, encompassing a decrease in recognition of peptidic epitopes. Immunized mice did not show significant levels of specific IgM and, after week 15, showed IgG titres lower than the infected mice. IgG1 was the predominant IgG subclass during all the experiment with background levels of IgG3. The mean Ab avidity was high and showed no significant changes during immunization. Different patterns of response were thus produced by dead and developing live parasites. Although high avidity IgG1 antibodies were early found in both cases, lower avidity IgG3 antibodies were increasingly produced afterwards only in infected animals. The isotype switch and avidity decrease observed only during infection are consistent with a possible parasitic mechanism to evade host immunity.*

Keywords E. granulosus, *secondary hydatidosis in mice, protoscoleces antigen, antibody avidity, IgG subclasses*

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INTRODUCTION

Infection by *Echinococcus granulosus* of its intermediate host produces cystic hydatid disease which is a chronic parasitic infection, generally showing no clinical symptoms and persisting for long periods of the host life. Immunity is thought to be most effective during the establishment phase of the infection. Once the metacestode (hydatid cyst) is established, the chronically infected host develops, in most cases, cellular and humoral specific responses. These immune responses are efficiently evaded by *E. granulosus* and do not protect the host against the established metacestode (Heath 1986, Craig 1988, Lightowlers 1990).

One possible mechanism leading to the production of less effective antibodies is the diversion of the response by carbohydrate antigens which has been described in other helminths (Maizels *et al*. 1993).

In the particular case of *Schistosoma mansoni*, glucidic epitopes have been proposed to induce in humans the synthesis of IgM and IgG2 that would block the effector functions of antibodies of other isotypes, potentially capable of mediating damage to the larvae (Butterworth *et al*. 1988, Langley & Dunne 1992). Data obtained in our laboratory suggest that *E. granulosus* carbohydrate epitopes are immunodominant both in natural (Sterla *et al*. 1997) and experimental hydatid infections (Ferragut & Nieto 1996, Míguez, Baz & Nieto 1996). In fact, anti-carbohydrate antibodies against hydatid cyst fluid antigen (HCFA), measured as those directed against periodate-sensitive moieties, may be as much as 66% of the specific serum antibodies in hydatid patients (Sterla *et al*. 1997) and 85% in experimentally infected mice (Ferragut & Nieto 1996). In addition, Ferragut & Nieto (1996), have observed that, during mouse experimental secondary infection, the maximum antibody response to HCFA peptidic epitopes coincides

with minimum anti-glucidic antibody response, suggesting some kind of interregulation of both types of response.

Carbohydrate antigens are known to be able to elicit T-independent (TI) antibody responses with no avidity maturation. TI antigens are divided into TI type 1 and type 2 antigens, and both categories induce predominantly IgM responses. TI type 1 antigens are usually mitogens, while carbohydrates with repeated molecular motifs are usually TI type 2 antigens.

In the mouse, in addition to IgM, TI type 1 antigens also stimulate IgG2a, IgG2b and IgG3 in similar amounts, whereas TI type 2 antigens stimulate predominantly IgG3 antibodies (Mond, Lees & Snapper 1995). Therefore, the study of antibody avidity as well as isotypic profile evolution during infection would be useful to analyze the type of antibody response elicited by carbohydrate antigens of *E. granulosus.*

Secondary hydatidosis established in mice by intraperitoneal inoculation of *E. granulosus* PSC has provided a useful model for the study of host-parasite relationships in hydatidosis (Heath 1970, Araj, Matossian & Frayha 1977, Cox, Marshall-Clarke & Dixon 1989, Dempster *et al*. 1992, Janssen *et al*. 1992, Liu, Lightowlers & Rickard 1992, Hernández & Nieto 1994, Ferragut & Nieto 1996, Míguez *et al*. 1996). Considering the mouse secondary infection as a mimic of what may happen after the surgical spillage in humans, the study of the response post-infection may provide useful information related to post-surgical relapse. Most of the immunological results reported in that model were obtained using HCFA as stage specific antigen. There is less information related to the antibody response against PSC antigens (Liu *et al*. 1992, Hernández & Nieto 1994, Haralabidis *et al*. 1995, Míguez *et al*. 1996). Besides this, comparison of specific antibody response elicited by live or integral dead PSC would give information about the role of the live parasite in the antibody response it elicits in the host during infection.

In this paper, we addressed the characterization of the antibody response against PSC somatic antigens (PSA) in mice either infected with viable PSC or immunized with integral dead PSC. Titres of antibodies recognizing peptidic and glucidic epitopes as well as their isotypes and avidities and the parasitic molecules they recognize in PSC were analysed weekly during 68 weeks, in both groups.

MATERIALS AND METHODS

Parasite antigen preparations

Protoscoleces (PSC)
PSC from bovine cysts were obtained as described by Ferragut & Nieto (1996).

Protoscoleces somatic antigen (PSA)
Antigen was prepared as described by Míguez *et al*. (1996).

Experimental design

Immunization and infection protocols were described in detail by Ferragut & Nieto (1996). Briefly, each of seventeen four-week-old Balb/c mice was infected by i.p. injection with a suspension of 2000 viable PSC in 0·5 ml of sterile PBS, containing 100 U/ml of Penicillin G and 100 μg/ml of Streptomycin. Similarly, three four-week-old Balb/c mice were i.p. immunized with a suspension of morphologically intact but non viable PSC, prepared by treatment of PSC with 70% ethanol.

Six uninoculated Balb/c mice served as sentinels.

Mice were weekly bled during 68 weeks and sera stored at −20°C. Pools were prepared by mixing equal volumes of sera from individuals of each group corresponding to the same extraction. All mice were weighed and autopsied on the 68th week (Ferragut & Nieto 1996).

Enzyme conjugated antibodies

One goat was immunized with mouse gammaglobulins according to Dresser (1986). Goat anti-mouse gammaglobulins were precipitated from that goat serum with 50% saturated ammonium sulphate, then reprecipitated with 40% saturated ammonium sulphate and extensively dialysed against PBS (Hudson & Hay 1989). Goat anti-mouse gammaglobulins were conjugated with peroxidase (Type VI-A, Sigma, St Louis MO, USA) according to Tijssen (1985).

Enzyme-linked immunosorbent assay (ELISA)

ELISA for determination of PSA-specific antibody titres was carried out coating polystyrene plates (Nunc, Denmark) with 25 μg/ml PSA following the protocol described by Hernández & Nieto 1994.

Total specific antibodies and specific IgG were titrated using either peroxidase-labelled goat anti-mouse immunoglobulins or peroxidase-labelled goat anti-mouse IgG (SIGMA, USA).

Specific antibodies of all IgG subclasses and IgM were titrated using mouse isotype-specific goat unlabelled antisera (NORDIC, The Netherlands) and peroxidase-labelled rabbit anti-goat IgG conjugate (SIGMA, USA) in subsequent steps.

Sodium metaperiodate treatment of native PSA

PSA fixed on nitrocellulose or coated on ELISA plates was treated with 20 mM sodium metaperiodate solution in 50 mM

sodium acetate buffer, pH 4·6, according to Woodward, Young & Bloodgood (1985).

Corresponding Western blot and ELISA techniques were performed as described by Hernández & Nieto (1994).

ELISA with thiocyanate elution for avidity determination

ELISA was performed as previously described (Pullen, Fitzgerald & Hosking 1986) with the following modifications. Each serum was diluted so as to give an $OD_{620} = 1{\cdot}00$ in ELISA and 100 μl/well were dispensed in eight wells. After three h incubation, supernatants were discarded, potassium thiocyanate was added to each of six wells of every sample at concentrations of 3·0, 2·5, 2·0, 1·5, 1·0 and 0·5 M in PBS-0·05% Tween 20 (PBS-T) (100 μl/well), and just PBS-T to the remaining two wells. Plates were incubated for 15 min to allow disruption of antigen-antibody interactions. The supernatants were discarded and, after washing, the corresponding conjugate (see ELISA) was added, and the ELISA completed as previously described.

SDS-PAGE and immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) using 12·5% acrylamide gels and 1% SDS. PSA was separated under reducing conditions (2% 2-mercaptoethanol) and electrotransferred onto nitrocellulose sheets (Bio-Rad, CA, USA) according to Towbin, Stahelin & Gordon (1979). The membranes were cut into strips which were blocked with 1% (v/v) Tween 20 in PBS for 30 min under rocking. Then, they were washed three times with PBS- 0·1% Tween 20 (PBS- 0·1%T) and once with PBS.

Blots were developed according to Farr & Nakane (1981). Serum samples were diluted in PBS-0·1%T and incubated for one h under rocking. After washing as above, the strips were incubated for one h with alkaline phosphatase-conjugated rabbit anti-mouse IgG and IgM (SIGMA, USA) appropriately diluted in PBS-0·1%T. Then, they were washed and substrate solution containing BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) and NBT (p-nitro-blue-tetrazolium chloride) was added according to the manufacturer's instructions (Bio-Rad).

Data analysis

Anti-PSA antibody concentration

A pool of serum samples from infected mice was used as reference serum. Antibody concentration expressed as arbitrary units (AU) and OD_{620} corresponding to dilutions of the reference serum were correlated by linear regression. ELISA data (OD_{620}) from every sample were converted to antibody concentrations equivalent to this reference for analytical consistency (Malvano *et al*. 1982).

Concentration of antibodies recognizing PSA carbohydrate epitopes

The concentration of antibodies recognizing periodate-treated PSA was considered as corresponding to anti-peptide (periodate resistant) antibodies. The decrease (%) in antibody concentration associated with periodate treatment provided an approximate estimation of the response to carbohydrate (periodate-sensitive) epitopes.

Concentration of anti-PSA antibodies of different isotypes

Titres of specific IgM, IgG and IgG subclasses were expressed as corresponding AU referred to the reference pool. AU corresponding to different isotypes were defined so that their values were the same for dilutions of the reference pool exhibiting similarly low values of OD_{620} (approx. 0·1) in each test.

In order to evaluate the contribution of each IgG subclass to total specific IgG, the ELISA results corresponding to each subclass expressed as AU were introduced as independent variables in multiple linear regression as a function of total specific IgG ($AU_{IgG} = aAU_{IgG1} + bAU_{IgG2a} + cAU_{IgG2b} + dAU_{IgG3}$). The coefficients (a, b, c, d) obtained from that multiple regression analysis were used to define new values of AU (AU') for each subclass to fit (F distribution; $P = 0{\cdot}05$) with the following equation: $AU'_{IgG} = AU'_{IgG1} + AU'_{IgG2a} + AU'_{IgG2b} + AU'_{IgG3}$.

Avidity index

The antibody concentration determined in the absence of thiocyanate was assumed to represent the total binding (100%) of specific antibodies, and those measured in the presence of decreasing concentrations of thiocyanate were converted to percentages of that total. The avidity index was calculated as the molar concentration of potassium thiocyanate required to reduce the maximum antibody concentration of each sample to 50%. Data were rejected as unreliable if the maximum value of OD_{620} was below 0·5.

Molecular weight determination in Western blot

Relative molecular weights (Mr) of PSA components recognized by mouse sera were estimated using a logarythmic plot of the relative migrations of a set of molecular weight standards included in every gel.

RESULTS

Mouse infection

All mice injected with viable PSC became infected. When

autopsied, they all contained hydatid cysts. (see Table 1 in Ferragut & Nieto 1996).

When mice injected with intact but non viable PSC were autopsied at the end of the protocol, PSC from the original inoculum were still observed at the site of injection.

Anti-PSA antibody response

Anti-PSA antibody titres were measured by ELISA during the whole course of the experiment (68 weeks) in pooled sera from each of the three groups of mice. These results are shown in Figure 1.

The infected mice sera showed significant Ab titres after week 3 and a steady titre increase up to week 38. From then up to week 53 titres remained high and then decreased until the end of the experiment. The immunized mice showed detectable levels of Ab after week 4. Nonetheless they show low and almost unchanged antibody titres along the protocol, the highest (five-fold lower than the corresponding infected mouse sera) being observed from week 38 to 58.

During infection the PSA-specific antibody response was predominantly against carbohydrate epitopes, $57 \pm 7\%$ at the beginning (week 3) up to $94 \pm 3\%$ at the end of the experiment. During immunization, carbohydrate epitopes were recognized by $85 \pm 11\%$ of the antibodies along the whole protocol (Figure 4).

Sera from sentinel mice did not show detectable anti-PSA antibodies.

Profiles of anti-PSA IgG and IgM

Anti-PSA IgG and IgM titre profiles corresponding to immunized and infected mice were followed from week 8 to 58. These results are shown in Figure 1.

Anti-PSA IgM and IgG showed similar patterns during infection, both of them peaking from week 38 to 50 (Figure 1a).

Concentrations of anti-PSA IgM in the sera from immunized mice were marginally above the background level of the negative controls (sentinels). On the other hand anti-PSA IgG in those mice peaked on week 15 (Figure 1b).

Profiles of anti-PSA antibodies of different IgG subclasses

The antibodies of the four IgG subclasses were studied from week 8 to week 58. The results obtained with the infected group are shown in Figure 2. Until week 18 post infection all subclasses of anti-PSA antibodies were similarly represented. After week 30 there was a marked increase of IgG3 titre which peaked on week 48. The titres of the other subclasses also peaked on week 48 but showed lower values.

The results obtained with the immunized group are shown in Figure 3. The titres of specific IgG subclasses in immunized mice showed similar patterns during all the experiment. IgG1 being the predominant subclass. All subclasses peaked on week 15 and showed titres comparable to those observed in corresponding sera from infected mice, up to week 25. After week 30, antibodies of all subclasses in immunized mice showed lower titres.

The ratio of specific IgG1/IgG3 changed during infection from a value higher than 10 at the week 6 to 0·5 at the end of the experiment (Figure 4). The same ratio did not have significant changes during immunization (7 ± 2) (Figure 4).

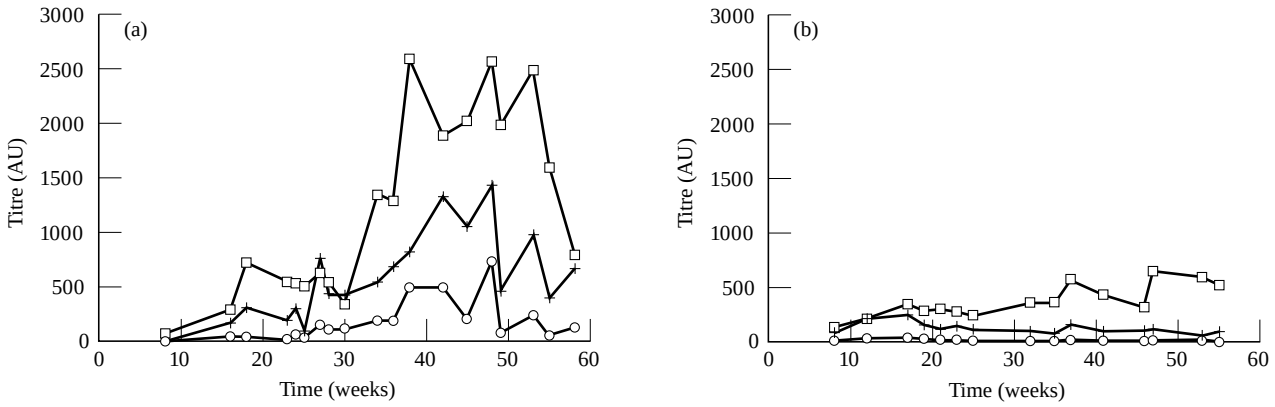

Figure 1 Titres of PSA-specific total immunoglobulins (■) as well as those of specific IgG (+) and IgM (○), determined by ELISA in pooled serum samples of infected (a) and immunized (b) mice, are shown in ordinates. Weeks after infection (a) or immunization (b) are shown in abcissae.

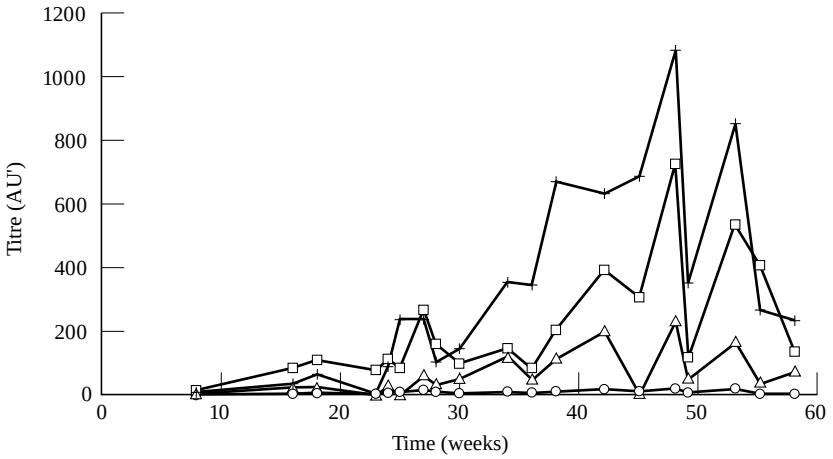

Figure 2 Titres of PSA-specific IgG1 (■), IgG2a (○), IgG2b (▲) and IgG3 (+) determined by ELISA in pooled serum samples of infected mice, are shown in ordinates. Titres are expressed in Au′ (see Data analysis) to allow comparison among subclasses and with IgG. Weeks after infection are shown in abcissae.

Evolution of antibody avidity

The patterns of evolution of antibody avidity corresponding to infected and immunized mice are shown in Figure 4.

Unexpectedly, a decrease of antibody avidity was observed after infection. The highest avidity index (2·7) was observed at week 6 after infection and a steady decrease was shown up to the end of the experiment (0·7).

Antibody avidity in sera from immunized mice was almost constant during all the experiment ($2{\cdot}1 \pm 0{\cdot}4$), and higher than in corresponding sera from infected mice.

The average avidities of PSA-specific IgG1 and IgG3, were $2{\cdot}1 \pm 0{\cdot}4$ and $1{\cdot}2 \pm 0{\cdot}2$, respectively, during the whole infection protocol. Those values were significantly different according to Student *t*-test ($a = 0{\cdot}05$).

Antigenic recognition of anti-PSA antibodies

Four pools were prepared using equal serum volumes from the 17 infected mice corresponding to the bleedings performed between weeks: 7–14 (pool 1), 20–29 (pool 2), 32–42 (pool 3) and 49–56 (pool 4). One pool was similarly prepared with sera from immunized corresponding to weeks 38–48 (pool 5).

These five pools were analysed by immunoblotting using native and periodate-treated PSA. A 60 kDa band was strongly recognized in native PSA by IgG from all the serum pools. This band was the less affected by periodate treatment. All the other bands disappeared with that treatment.

The IgM antibodies in all pools reacted only with native PSA, and complete lack of reaction was observed in the cases of pool 1 and pool 5.

DISCUSSION

Our results show that the PSA-specific antibody response after mouse ip infection with *E. granulosus* PSC was strong

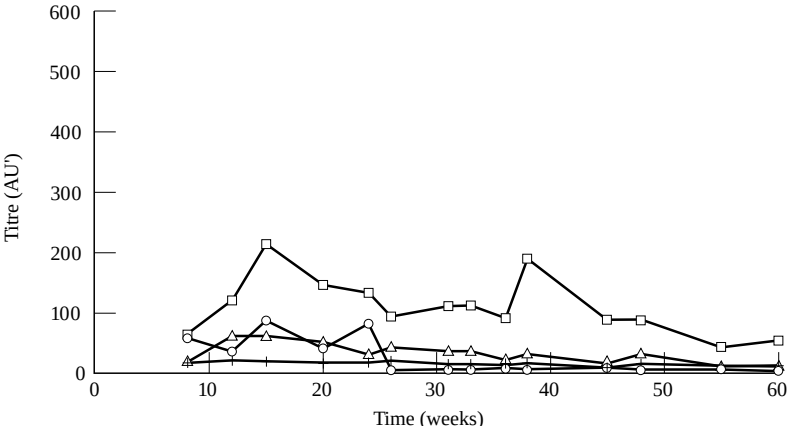

Figure 3 Titres of PSA-specific IgG1 (■), IgG2a (○), IgG2b (▲) and IgG3 (+) determined by ELISA in pooled serum samples of immunized mice, are shown in ordinates. Titres are expressed in Au′ (see Data analysis) to allow comparison among subclasses and with IgG. Weeks after immunization are shown in abcissae.

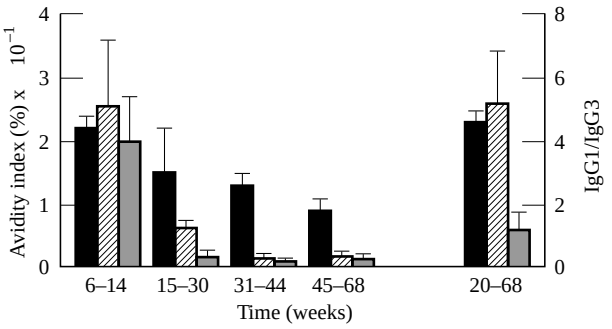

Figure 4 Avidity indexes of PSA-specific antibodies (solid bars), and the percentages of PSA-specific antibodies recognizing peptidic epitopes (toned bars) are both represented in the left hand side ordinates axis. The figures represent the actual values of avidities indexes and one tenth of the anti-peptide antibodies percentages ($(\%)\times10^{-1}$). In the right hand side axis are represented the ratios of PSA-specific IgG1 to IgG3 titres (crossed bars). Equal volumes of serum samples extracted weekly from each mouse in each experimental group during the period of time (weeks) represented in abcissae, were pooled to analyse avidity, IgG1/IgG3 ratio and percentage of anti-peptide antibodies. In the case of the immunized group, as there were no significant differences along the protocol, the average values are shown on the '20–68' abcissae.

and predominantly against carbohydrate epitopes (Figure 4). This effect was more evident after infection that after administration of dead PSC (immunization) showing up to $94 \pm 3\%$ and $85 \pm 11\%$ of anti-carbohydrate antibodies respectively.

Anti-PSA antibodies appeared earlier (Figure 1), and showed only minor fluctuations with time compared with anti-HCFA antibodies (Ferragut & Nieto 1996). These results are in agreement with those reported by Liu *et al*. (1992).

An IgG3 MoAb (E492) prepared in our laboratory and directed against a glucidic epitope in PSA (Bentancur *et al*. 1996), was used to purify by affinity cromatography the corresponding glycoconjugates from PSA. The Ab response against this purified antigen showed a similarly shaped profile as that shown by the Ab response to periodate-treated PSA (data not shown) suggesting that this treatment provides data that correlates with anti-carbohydrate antibody response. Our results show that immunization with dead *E. granulosus* PSC induces a stable PSA-specific antibody response characterized by higher concentrations of IgG1 (Figure 3). On the other hand, *E. granulosus* infection induces high levels of IgG1 during early stages, but later on generates higher levels of IgG3 and IgM (Figure 2) which were not observed after immunization. The titres of PSA-specific immunoglobulins as well as PSA-specific IgG before week 25 were similar in the immunized and infected groups of mice. Afterwards the infected group showed higher titres than the immunized group. The same profile was observed in the case of specific IgG1 titres. Although immunogenicity of ethanol-treated PSC may be different from that of *in vivo* killed PSC, the observed similarity between both experimental groups at the beginning of the experiment is consistent with the fact that more than 90% of the PSC used for infection actually died (Ferragut & Nieto 1996). IgG2a and IgG2b did not show significant changes during immunization and infection. After infection with brood capsules (50 PSC of *E. granulosus* per mice) Haralabidis *et al*. (1995), found a similar pattern of response up to the week 30. In their study no sample was taken between weeks 30 and 68 after infection, the period when we observed the maximum levels of anti-PSA IgM and IgG.

Other results obtained in our laboratory have also shown a relevant anti-carbohydrate antibody response in hydatid patients (Sterla *et al*. 1997). Moreover, anti-PSA specific IgG2 was observed to be dominant in the sera of hydatid patients (Sterla, personal communication). These results are in agreement with ours, as murine IgG3 is proposed to be a functional analogue of human IgG2 (Perlmutter *et al*. 1978).

The avidity of PSA-specific immunoglobulins showed an unexpected profile, the highest values being observed at early stages of infection (Figure 4). The avidity of PSA-specific IgG antibodies showed a similar profile (data not shown), consistently with the expected constant low avidity of PSA-specific IgM. However, avidities of PSA-specific antibodies of individual IgG subclasses remained almost constant during infection. Average avidity values of IgG1 were similar to the mean IgG avidity in early infection while the values of the IgG3 avidities were lower and similar to the mean IgG avidity observed at week 50. Interestingly, we also observed that avidities of immunoglobulins recognizing PSA peptidic epitopes were similar to those of PSA-specific IgG1, while PSA-specific total immunoglobulins showed lower avidities (data not shown).

In addition, the ratio IgG1/IgG3 was lower at week 50 than at the beginning of infection but remained constant during immunization. There was a clear change of this ratio around week 25 of infection which is coincident with the appearance of cyst fluid-specific anti-carbohydrate antibodies (Ferragut & Nieto 1996). These results suggest that some molecules excreted/secreted by the living parasite may be down-regulating the anti-peptide specific high avidity IgG1 response and up-regulating an anti-carbohydrate specific low avidity IgG3 and IgM response.

Somatic PSC antigens were available to the immune system of both groups of mice, according to the presence of PSC observed at the site of injection when the immunized mice were autopsied. Therefore, the difference in PSA-specific antibody responses elicited by infected and immunized mice may be associated mainly with the products excreted/secreted by live PSC.

We observed a significant decrease in PSA-specific Ab response in the last weeks of the experiment. Similar results were observed when anti-HCFA Ab response was studied (Ferragut & Nieto 1996). Therefore this decreased PSA-specific response coexists with a decrease also in Ab response to cyst antigens, thus suggesting it is not a stage specific decrease, but rather a general decrease in Ab response to the parasitic antigens.

Western blot results (Figure 5) were consistent with the ELISA ones. They showed that all the antigens recognized by IgM and almost all those by IgG, during all the infection protocol, were carbohydrates. Only a 60 kDa band appeared to have periodate-resistant epitopes recognized by IgG. Interestingly that band was recognized by both infected and immunized mice.

The decrease in the ratio of IgG1/IgG3 titres observed only in infected mice may explain the declining profile of total specific antibody avidity, observed during infection but not during immunization.

Mice infected with *Trypanosoma cruzi* also showed high IgM titres during chronic disease, and also IgG1 was the subclass showing the highest avidity. However, differently from the case of *E. granulosus*, avidity maturation was observed in the case of *T. cruzi* infection (El Bouhdidi *et al*. 1994).

Carbohydrate antigens may elicit a T-independent antibody response. In particular, the type 2 T-independent antigens elicit a mouse antibody response strongly restricted to IgM and IgG3 isotypes (Mond *et al*. 1995). In other parasitic infections, in particular with *S. mansoni*, it has been shown that the response against carbohydrate-rich egg antigens is predominantly of IgG3 and IgM isotypes (Dunne 1990, Mazza, Dunne & Butterworth 1990, Langley & Dunne 1992) and this has been proposed as an evasion mechanism. Moreover, proliferation of IgM- and IgG3-producing B1 cells induced by specific *S. mansoni* carbohydrates has been described *in vitro* (Velupillai & Harn 1994) and *in vivo* (Velupillai, Sypek & Harn 1996).

Our results show that *E. granulosus* chronically infected mice produce a strong low avidity antibody response to parasitic carbohydrates, predominantly of IgG3 and IgM isotypes.

The characteristics of this response suggest it may have a relevant T-independent type 2 component that cannot be associated with efficient effector mechanisms, thus suggesting it may be involved in a parasite mechanism to evade host immunity.

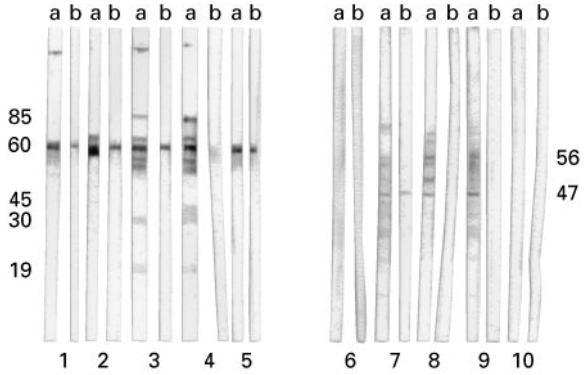

Figure 5 Reaction with reduced native (a) and periodate-treated (b) PSA of pooled serum samples weekly extracted from mice at different times during infection and immunization protocols are shown in different lanes according to the following scheme, as well as the serum dilution used: Lanes 1 and 6: Pool of infected mice sera from weeks 7–14 (1/100); Lanes 2 and 7: Pool of infected mice sera from weeks. 20–49 (1/100); Lanes 3 and 8: Pool of infected mice sera from weeks 32–42 (1/400); Lanes 4 and 9: Pool of infected mice sera from weeks 49–56 (1/400); Lanes 5 and 10: Pool of immunized mice sera from weeks 38–48 (1/100); Lanes 1 to 5 correspond to the reaction of IgG antibodies and lanes 6 to 10 correspond to that of IgM antibodies.

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