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Antibody response in CD4-depleted mice after immunization or during early infection with *Echinococcus granulosus*

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SUMMARY

The aims of this work were to investigate the existence of T-independent antigens in Echinococcus granulosus *protoscoleces and to evaluate the relative contribution of T-independent stimulation to the overall antibody response in early infection. Mice depleted of $CD4^+$-cells were immunized with protoscolex somatic antigens (PSA) or infected with* E. granulosus *protoscoleces (PSC). Results showed that the response of CD4-depleted immunized mice had the expected characteristics of a T-independent stimulation and that such T-independent stimulation was important mainly during primary response. During infection absence of $CD4^+$-cells affected mainly the secretion of all IgG subclasses with the exception of IgG3 and IgM. To carry out a preliminary isolation of PSC T-independent antigens we prepared a carbohydrate enriched fraction from protoscolex antigens, using a monoclonal antibody specific for the carbohydrate moiety Galα(1,4)Gal highly expressed in PSC. This fraction was mitogenic for naive mouse splenocytes and was recognized by a high percentage of the specific antibodies secreted by CD4-depleted immunized or infected mice. In summary, these results suggest that* E. granulosus *protoscoleces contain immunogenic T-independent antigens. Primary antibody responses to protoscolex somatic antigens and the production of IgM and IgG3 in early infection would be mainly stimulated by a T-independent mechanism.*

Keywords Echinococcus granulosus, *hydatid disease, T-independent antigens, $CD4^+$-T cells, parasite carbohydrate antigens*

INTRODUCTION

Evidence has accumulated demonstrating that pathogen carbohydrates possess important biological activities, including mimicry of host components (Mandrell *et al*. 1992), association with pathogenicity (Rasool *et al*. 1992), inhibition of cell proliferation (Persat *et al*. 1996) and inhibition of the macrophage effector function (Vecchiarelli *et al*. 1995). Pathogen carbohydrates, as antigens, can also drive Th1/Th2 polarization (Velupillai & Harn 1994), induce protection (Ellis *et al*. 1994), stimulate production of blocking antibodies (Butterworth *et al*. 1988) and promote autoimmune phenomena (Galili *et al*. 1988). Many bacteria, fungi, viruses and parasites possess immunogenic carbohydrates which can induce T-independent immune responses (Mazza *et al.* 1990, Ishioka *et al*. 1993, Mond *et al*. 1995) and nonspecific polyclonal activation (Jenkins *et al*. 1986, Judson *et al.* 1987, Cox *et al.* 1989, Schoenfeld *et al*. 1992, Honarvar *et al*. 1994).

Carbohydrates are important components of *Echinococcus granulosus* protoscoleces, cyst fluid and laminated layer (McManus & Bryant 1995). Studies in both the mouse experimental model and infected humans have demonstrated that protoscolex or cyst carbohydrate epitopes are immunodominant (Hernández & Nieto 1994, Ferragut & Nieto 1996, Miguez *et al*. 1996, Severi *et al.* 1997, Sterla *et al.* 1997). Chronically infected mice produce mainly IgG3 and IgM antibodies against protoscolex carbohydrates (Severi *et al.* 1997). Lack of avidity maturation and an important recognition of carbohydrate epitopes from hydatid cyst fluid antigens are also features of the antibody response in chronic experimental infection (Ferragut & Nieto 1996). Severi *et al*. (1997) suggested the existence of T-independent immunogenic carbohydrates in *E. granulosus* protoscoleces, and that the stimulation of a T-independent response could be involved in the evasion of host immunity. However, no direct evidence is available for the existence of T-independent antigens in *E. granulosus* nor for the importance of a T-independent response in immunization or infection.

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In the present study we have used *in vivo* depletion of $CD4^+$-cells in mice as an experimental model for the analysis of T-independent responses. The first aim was to investigate the existence of T-independent antigens in *E. granulosus* protoscoleces. For this purpose we analysed the production of specific antibodies in CD4-depleted mice immunized with protoscolex antigens.

The second aim was to evaluate the relative contribution of the T-independent stimulation to the overall antibody response in early infection. For this purpose, we characterized the antibody response of CD4-depleted mice infected intraperitoneally with protoscoleces.

We focused on the study of early infection since a profound knowledge of the immune response very early in infection may be crucial for the design of immunointervention strategies in human secondary infection. We believe so, because once protoscoleces differentiate into cysts, laminar and adventitial layers act as barriers impairing the access of effector cells to the germinal layer (Thompson 1995) therefore, the metacestode seems to be less exposed to the immunological attack than protoscoleces.

MATERIALS AND METHODS

Mice

BALB/c mice were obtained from Charles River Breeding Laboratories, Inc. (Wilmington MA, USA) and subsequently bred at the Instituto de Higiene (Montevideo, Uruguay). Mice were tested by coprological analysis to confirm that they were parasite-free.

Antigens

Hydatid cyst fluid (HCFA) and viable protoscoleces (PSC) were obtained from bovine hydatid cysts according to Baz *et al*. 1995. Protoscolex somatic antigens (PSA) were prepared by sonication of viable parasites as described previously (Miguez *et al*. 1996). Hydatid cyst wall antigens (HCWA) were prepared as described previously (Khoo *et al*. 1997) by sonication of bovine hydatid cyst walls at 4°C in the presence of 2 mM phenylmethylsulphonyl fluoride (Sigma) and EDTA at maximum power with an ultrasonic disruptor (Blackstone, USA). Insoluble material was separated by centrifugation at 8000 ***g***, and supernatants stored at – 20°C. Excretion/secretion protoscolex antigens (E/S), were obtained by culture of viable protoscoleces in RPMI at 37°C in 5% CO_2 for 20 days. Supernatants were recovered and fresh medium was added every two days. All supernatants were dialysed against Milli-Q water, freeze-dried, resuspendend in phosphate buffered saline (PBS) and stored at – 20°C. All antigens were filtered through a 0·22-μm membrane before use.

Monoclonal antibodies

Preparation of E492/G1 monoclonal antibody

Monoclonal antibodies were produced by standard methods (Liddell & Cryer 1991a). Adult BALB/c mice were immunized with protoscolex somatic antigens as follows: 50 μg of PSA in Complete Freund's Adjuvant (CFA) i.p. on day 0; 50 μg of PSA in Incomplete Freund's Adjuvant (IFA) i.p. on days 30 and 60; and 100 μg of PSA in PBS i.v. on day 90. On day 93, splenocytes were fused with X63 (ATCC) myeloma cells. Screening was carried out by ELISA on PSA coated microtiter plates (Miguez *et al*. 1996). Isotype analyses were performed using goat anti-(mouse IgM) or goat anti-(mouse IgG) conjugated to peroxidase (Sigma) and the reaction was developed with 3-methyl-2-benzothyazolinona (MBTH), 3-dimethyl-amino benzoic acid (DMAB) and H_2O_2. Optical density at 600 nm was measured in an ELISA reader (Multiskan, Titertek, Labsystem). Hybridoma-secreting PSA-specific antibodies were tested for the ability to recognize periodate sensitive epitopes by ELISA (Woodward *et al.* 1985). Cloning was performed four consecutive times by limiting dilution, and confirmed by determination of the isoelectric point of the secreted monoclonal antibody (Liddell & Cryer 1991b). A cloned hybridoma (E492/G1) was further characterized by ELISA on native and periodate treated PSA, HCFA, HCWA and E/S. The IgG subclass of E492/G1 was determined by double diffusion in gel using mouse anti-IgG1, -IgG2a, -IgG2b or -IgG3 antibodies (Nordik). The pattern of PSA components recognized by E492/G1 was analysed by Western blot as described below. The epitope recognized by E492/G1 was further characterized by ELISA on plates sensitized with different disaccharides coupled to human serum albumin (HSA). The monoclonal antibody reacted with globotriose-HSA so the epitope was further confirmed by inhibition ELISA using the disaccharides Galα(1,4)Gal; Galβ(1,4)Glc and Galα(1,6)Glc as inhibitors (BioCarb Chemicals).

Anti-mouse L_3T_4 monoclonal antibody

The rat hybridoma GK 1·5 (antimouse L_3T_4, ATCC) was grown in RPMI 1640 supplemented with 10% Foetal Calf Serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate (all reagents from Sigma) at 37°C in 5% CO_2. The monoclonal antibody antimouse L_3T_4 was purified from culture supernatants by affinity chromatography on Protein-G (Pharmacia, Sweden) according to the manufacturer's instructions, dialysed against PBS and filtered through a 0·22-μm membrane.

Purification of protoscolex antigens recognized by E492/G1

Protoscolex somatic antigens were adsorbed onto E492/G1-CNBr Sepharose. The retained fraction was eluted with Gly-HCl 0·1 M, pH 2·8 and collected into 2 M Tris pH 11. The eluted fraction ($E4^+$) was dialysed against Milli-Q water and freeze-dried. Antigens were resuspended with PBS, filtered through a 0·22-μm membrane, and protein and carbohydrate contents were determined as described below.

Protein content

Protein contents of antigenic preparations were determined by the bicinchoninic acid method (BCA Protein Assay Reagent, Pierce, IL, USA) using bovine serum albumin as standard, according to the manufacturer's instructions.

Carbohydrate content

Carbohydrate contents of antigenic preparations were determined by a modified orcinol-sulphuric acid assay (White & Kennedy 1986) using resorcinol according to Miguez *et al*. (1996).

Determination of mitogenic activity of parasite antigens

Naive BALB/c splenocytes (2×10^5/well) were cultured in complete medium (RPMI 1640; 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 50 μM mercaptoethanol (all reagents from Sigma), with different concentrations of PSA or $E4^+$ for 48 h in triplicates. LPS (Sigma) at 12 μg/ml was used as positive control of mitogenic activity. Different concentrations of bovine IgG (Sigma) were used as a negative control of mitogenic activity. After incubation, cells were pulsed with 1 μCi/well of [^{3}H]-thymidine (Amersham) and incubated for 16 h before harvesting. Thymidine incorporation was assessed by liquid scintillation spectrometry (Beckman LS 6000). The stimulation index (SI) for each antigen was calculated as the mean cpm of stimulated cells/mean cpm of nonstimulated cells. The amount of carbohydrates from PSA or $E4^+$ per well that induced the greatest SI was determined. These data were used to calculate the specific mitogenic activity (SMA) for each antigen as follows:

SMA = SI/μg of carbohydrates added per well

In vivo $CD4^+$-cell depletion

Adult *BALB/c* mice were treated intraperitoneally with 320 μg of antimouse L_3T_4 in PBS for three consecutive days (day 1, 2 and 3 of experiment) followed by weekly doses of 800 μg (CD4-depleted group). A second group of mice injected with PBS was used as negative control for depletion (PBS-treated group). In a first experiment depletion was confirmed in spleen cells and peripheral blood cells by immunofluorescence with anti-L_3T_4 or anti-Lyt2 monoclonal antibodies conjugated to biotin (Pharmingen) and streptavidin-FITC (Amersham) as second reagent. Unrelated monoclonal antibodies of identical isotypes were included in this assay. In the second experiment (immunization) and in the third experiment (infection), depletion was controlled in peripheral blood cells periodically for each mouse, to verify that $CD4^+$-cells remained depleted until the end of each experiment.

Immunizations

A group of CD4-depleted mice (CD4-depleted immunized mice) and another group of PBS-treated mice (control immunized mice), were primed i.p. on day 8 with 100 μg of PSA in CFA and boosted i.p. on day 36 with 50 μg of PSA in IFA. In addition, PBS-treated or CD4-depleted mice inoculated with CFA or IFA alone were used as negative controls for the immunization (unimmunized mice). All mice were periodically bled and sera were stored at – 20°C.

Infections

A group of CD4-depleted mice (CD4-depleted infected mice) and another group of PBS-treated mice (control infected mice) were infected with 2000 viable PSC per mouse in PBS, i.p. (Baz *et al*. 1995). Other groups of CD4-depleted or PBS-treated mice were inoculated with PBS alone as negative controls for the infection (uninfected mice). All mice were bled over a period of 7 weeks and sera were stored at – 20°C.

Determination of specific antibodies in serum

Determination of anti-PSA antibodies in all sera was done by ELISA (Miguez *et al*. 1996). Specific IgG and specific IgM were determined using rabbit anti-(mouse IgG) (Sigma) or rabbit anti-(mouse IgM) labelled with peroxidase (Sigma), respectively. Specific IgG subclasses were determined according to Severi *et al*. (1997), using appropriate dilutions of goat anti-(mouse IgG1, -IgG2a, -IgG2b or -IgG3) (Nordik, UK), and rabbit anti-(goat IgG) antibodies coupled to peroxidase (Sigma). A pool of positive sera was used as standard in all assays and optical densities at 600 nm (OD 600) measured for each sample were converted to arbitrary units (AU) equivalent to this reference (Malvano *et al*. 1982).

Inhibition ELISA

Inhibition of antibody binding to PSA by the fraction $E4^+$ was tested in an ELISA protocol similar to that described above except that samples were incubated with different concentrations of $E4^+$ for one h at 37°C before being added to the ELISA plates. The percentage inhibition was calculated for each concentration of $E4^+$ according to the equation:

Percentage inhibition $= [(OD_0 - OD_i)/OD_0] \times 100$

OD_i: OD of samples with inhibitor

OD_0: OD of samples without inhibitor

SDS-PAGE and immunoblotting

PSA was resolved by SDS-PAGE (Laemmli 1970) in 10% w/v polyacrylamide gels under reducing conditions and electrophoretically transferred onto nitrocellulose sheets (BioRad, CA, USA) according to Towbin *et al.* (1979). Immunoblotting was performed as previously described (Severi *et al*. 1997) using goat anti-(mouse IgG) conjugated to alkaline phosphatase (Sigma) and developed with BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) and NBT (p-nitro-blue-tetrazolium chloride) (BioRad).

Sodium metaperiodate treatment of native antigen

PSA transferred onto nitrocellulose was treated with sodium m-periodate solution according to Hernández & Nieto (1994). The immunoblotting were similar to those described above.

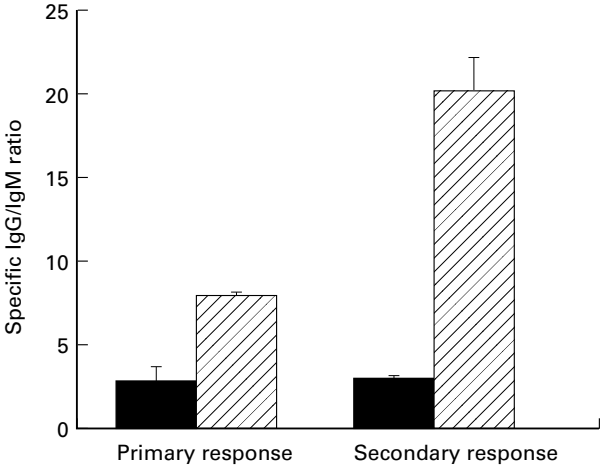


Figure 1 Specific anti-PSA IgG/IgM ratio of CD4-depleted immunized mice (solid bars) and control immunized mice (ribbed bars). Titres of IgG and IgM anti-PSA were determined by ELISA in sera of individual mice ($n = 4$) as described in Methods and specific anti-PSA IgG/IgM ratio was calculated for each individual mouse. Bars represent mean ± SEM for both groups of mice in primary and secondary responses.

Immunohistochemistry

Viable protoscoleces fixed in 10% buffered formalin were embedded in paraffin following standard protocols. Sections (5 μm) were dewaxed, rehydrated, treated with 0·1% H_2O_2 for 15 min and then incubated for one h at 37°C with E492/G1 or with an unrelated IgG3 monoclonal antibody (Nordik). This was followed by incubation with rabbit anti(mouse IgG) antibodies coupled to peroxidase (Sigma) for one h at 37°C. To develop the reaction 3,3′-diaminobenzidine and H_2O_2 were used.

Statistics

Data obtained from each group were compared by Student's *t*-test. Differences at $P < 0{\cdot}05$ were regarded as significant.

RESULTS

Effect of $CD4^+$–cell depletion on anti–PSA antibody responses after immunization

Treatment of mice with anti-L_3T_4 monoclonal antibody induced a depletion of $79 \pm 7\%$ of the $CD4^+$-cells in spleen and peripheral blood. In CD4-depleted immunized mice the booster dose of PSA did not result in a sharp increase in antibody titres, or in an increase in the specific IgG/IgM ratio (3 ± 2 both after priming and after boosting) (Figure 1). In contrast, in control immunized mice a clear booster effect was indicated by an increase in the specific IgG/IgM ratio from 7 ± 1(after priming) to 20 ± 4 (after boosting). Lack of $CD4^+$-cells mainly affected the secretion of specific IgG during secondary response. Mean primary IgM and IgG titres in CD4-depleted immunized mice reached 100% and 60% of those observed in control immunized mice, respectively. However, in secondary response these values decreased to 15% for IgM and 2% for IgG.

Elimination of $CD4^+$-cells affected mostly the production of antibodies to peptidic epitopes since sera from CD4-depleted immunized mice only recognized periodate sensitive epitopes in contrast to control immunized mice (Figure 2). Analysis of sera from CD4-depleted immunized mice by western blot demonstrated that immunogenic T-independent antigens are mainly molecules of relative molecular weight in the range from 67 to 40 kDa. A similar analysis of sera from control immunized mice showed that the response to carbohydrate epitopes was also localized within the same MW range (Figure 2; compare 1B with 2B).

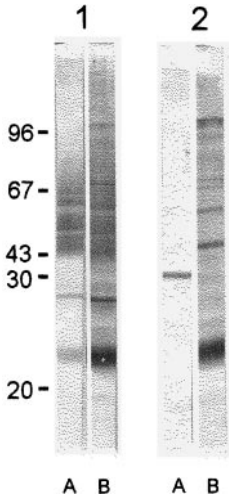


Figure 2 Antigen fractions recognized by sera from both CD4-depleted immunized mice (A) and control immunized mice (B), studied by Western blot. Analysis was performed on day 28, on native PSA (1) or periodate treated PSA (2).

Effect of CD4$^+$–cell depletion on anti–PSA antibody responses during infection

As expected, CD4-depleted infected mice produced significantly lower levels of specific IgG than control infected mice (Figure 3b). In contrast, specific IgM production was not affected by CD4$^+$-cell depletion (Figure 3a). Consistent with the expected features of a T-independent response, sera from CD4-depleted infected mice reacted mainly with periodate-sensitive epitopes, in contrast to what was found in control infected mice (Figure 4).

When the specific IgG response was dissected into the different subclasses it was observed that IgG3 was the only subclass detected in sera from CD4-depleted infected mice. In contrast, control infected mice produced all IgG subclasses. Interestingly, the levels of IgG3 detected in the sera from both infected groups were similar (Figure 5).

A carbohydrate rich fraction from protoscoleces containing putative T-independent antigens

Monoclonal antibodies were produced from mice immunized with PSA and their specificity was analysed by ELISA on different *E. granulosus* antigens. A particular antibody (E492/G1, isoelectric point 8, IgG3 isotype) was found to recognize a periodate-sensitive epitope present in protoscolex somatic antigens, hydatid cyst fluid antigens and hydatid cyst wall antigens, but weakly in excretion/secretion antigens (Table 1). In protoscoleces, the epitope was widely distributed being present in many components of protoscolex somatic antigens separable on SDS-PAGE (Figure 6). The MoAb E492/G1 was shown to recognize by ELISA

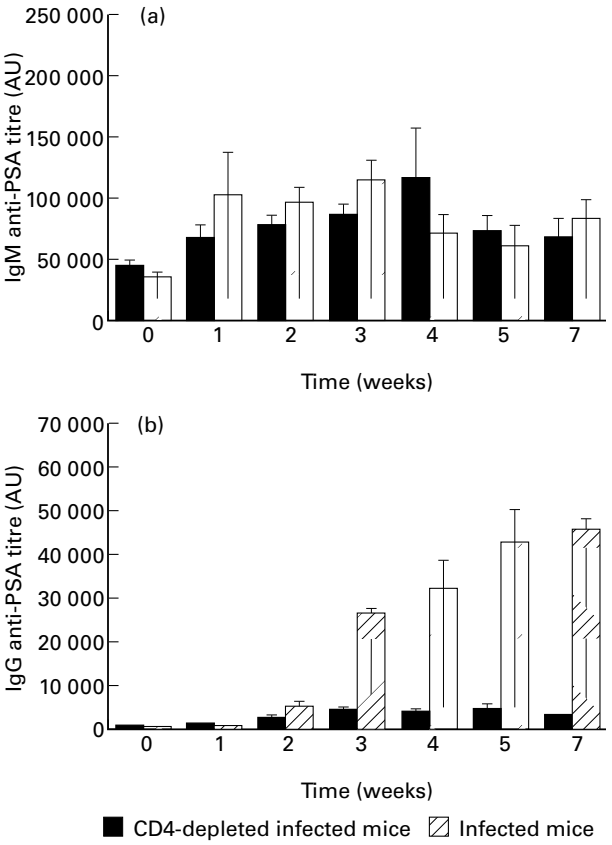


Figure 3 Specific anti-PSA IgM titres (a) and specific anti-PSA IgG titres (b) of CD4-depleted infected mice (solid bars) and control infected mice (ribbed bars). Results are expressed in arbitrary units of concentration for IgG and IgM. Titres were determined by ELISA in individual sera and expressed as mean ± SEM for seven mice. Background titres for uninfected mice were less than 45 000 AU for IgM and 1000 AU for IgG.

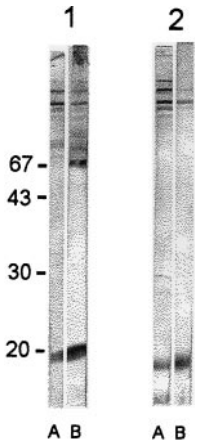


Figure 4 Antigen fractions recognized by sera from CD4-depleted infected mice (B) and control infected mice (A), studied by Western blot. Analysis was performed at week 7 postinfection on native PSA (1) and periodate treated PSA (2).

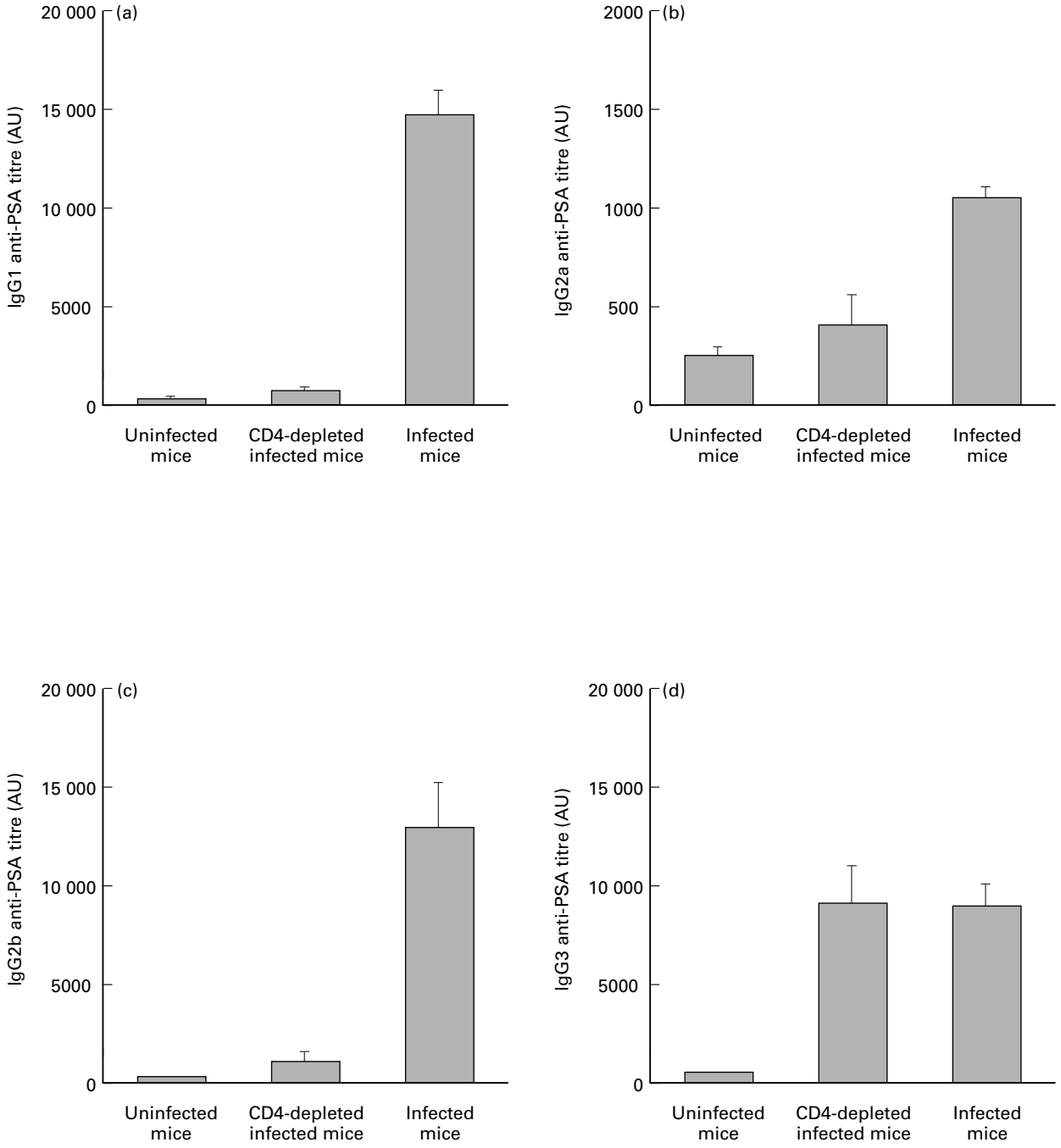


Figure 5 Antibody titres from CD4-depleted infected mice and control infected mice. Results are expressed in arbitrary units of concentration for anti-PSA specific IgG1 (a), IgG2a (b), IgG2b (c) and IgG3 (d). The analysis was performed when IgG titres reached a maximum (week 7 postinfection). Titres were determined by ELISA in individual sera and expressed as mean ± SEM for seven mice.

globotriose coupled to human serum albumin (HSA) but not melibiose-HSA. The specificity of the recognition was confirmed by inhibition ELISA using synthetic disaccharides. Only the disaccharide Galα(1,4)Gal, but not Galα(1,6)Glc nor Galβ(1,4)Glc, was able to inhibit the binding of E492/G1 to globotriose-HSA (a maximal value of percentage inhibition of 80% with 10 μg per well of the disaccharide Galα(1,4)Gal was found).

The components of PSA recognized by E492/G1 were purified by antibody affinity chromatography using the

Table 1 Antigenic specificity of E492/G1 monoclonal antibody

	O.D. (600nm)			
	PSA	E/S	HCWA	HCFA
E492/G1 culture supernatant	0·82 ± 0·17	0·17 ± 0·01	0·83 ± 0·12	0·30 ± 0·01
Negative culture supernatant	<0·01	<0·01	<0·01	<0·01
Positive control	0·94 ± 0·07	0·47 ± 0·19	0·95 ± 0·06	0·43 ± 0·05

Culture supernatants were tested by ELISA on plates sensitized with PSA, E/S, HCWA or HCFA. Antigens were prepared as is described in Material and Methods. The serum from the immunized mice used to obtain the hybridoma E492/G1 was used as positive control. Supernatants from hybridoma producing antibodies nonspecific for PSA, E/S, HCWA or HCFA were used as negative controls. When sensitized plates were treated with sodium m-periodate to eliminate carbohydrate epitopes optical densities were less than 0·01 in all cases. Results are expressed as the mean ± standard deviation of the optical densities obtained from two independent assays.

immobilized monoclonal antibody, to yield a fraction ($E4^+$) which had a carbohydrate/protein content ratio of 4 : 1 while for PSA the same ratio was of 1 : 1.

The fraction $E4^+$ induced a proliferative response in naive mouse spleen cells. The greatest stimulation indices obtained for $E4^+$ and PSA in three independent experiment were 4 ± 1 and 5 ± 2, respectively. Under the same conditions, the positive control (LPS) and the negative control (bovine IgG) yielded values of 20 ± 3 and $1{\cdot}4 \pm 0{\cdot}2$, respectively. Specific mitogenic activity, defined as the ratio of the greatest stimulation index per μg of carbohydrate added per well obtained from three independent experiments, was $1{\cdot}7 \pm 0{\cdot}5$ for $E4^+$ and $0{\cdot}5 \pm 0{\cdot}2$ for PSA.

Given that $E4^+$ contained a widely distributed carbohydrate epitope (Figure 6) which was associated with immunogenic antigens, the possibility was tested that $E4^+$ components may be involved in eliciting a T-cell independent antibody response. This was performed by analysing whether antibodies from CD4-depleted immunized mice, were able to recognize $E4^+$ by inhibition ELISA. Results showed that the $E4^+$ fraction could inhibit $38 \pm 3\%$ and $37 \pm 10\%$ of the specific IgM and IgG responses, respectively, to PSA (Figure 7a). The relevance of the T-independent production of $E4^+$-specific antibodies was similarly evaluated in sera of normal and CD4-depleted infected mice. Results showed that $E4^+$ inhibited $32 \pm 7\%$ of the specific IgM produced on day 7 postinfection in sera from CD4-depleted mice. A similar percentage inhibition was observed with sera from control infected mice ($48 \pm 5\%$) (Figure 7b). Inhibition of specific IgG was not performed because IgG titres were too low in CD4-depleted infected mice.

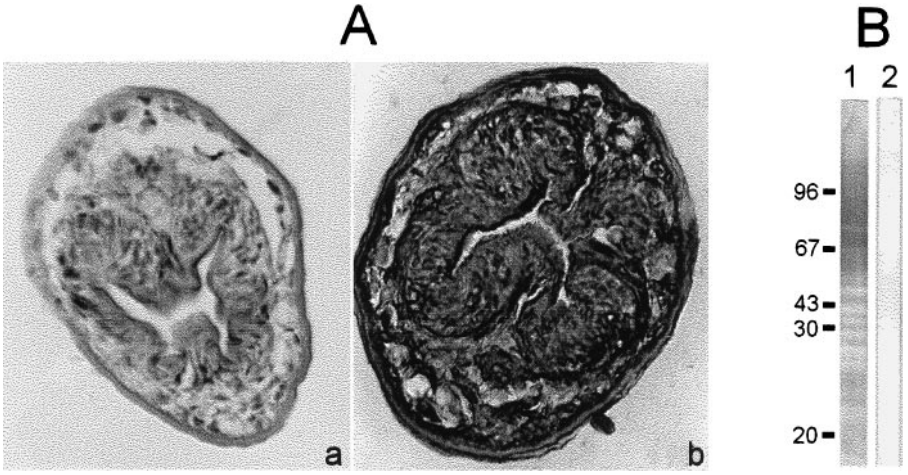
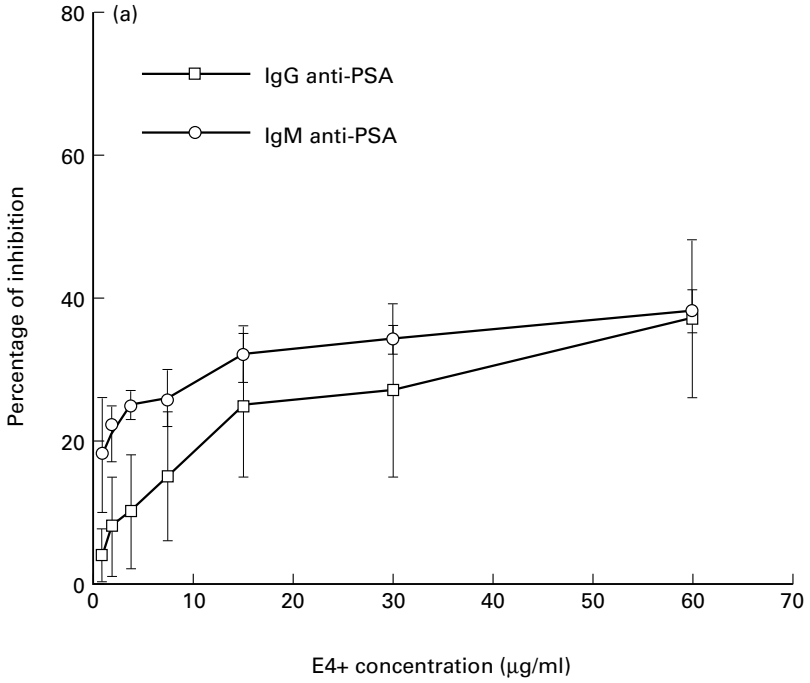


Figure 6 A: Histological localization of the epitope recognized by E492/G1 on *E. granulosus* protoscolex sections (b). A nonrelated IgG3 monoclonal antibody was used as a negative control (a). B: Protoscolex somatic antigens recognized by the monoclonal antibody E492/G1, studied by Western blot are shown. Native PSA (lane 1) and periodate treated PSA (lane 2).



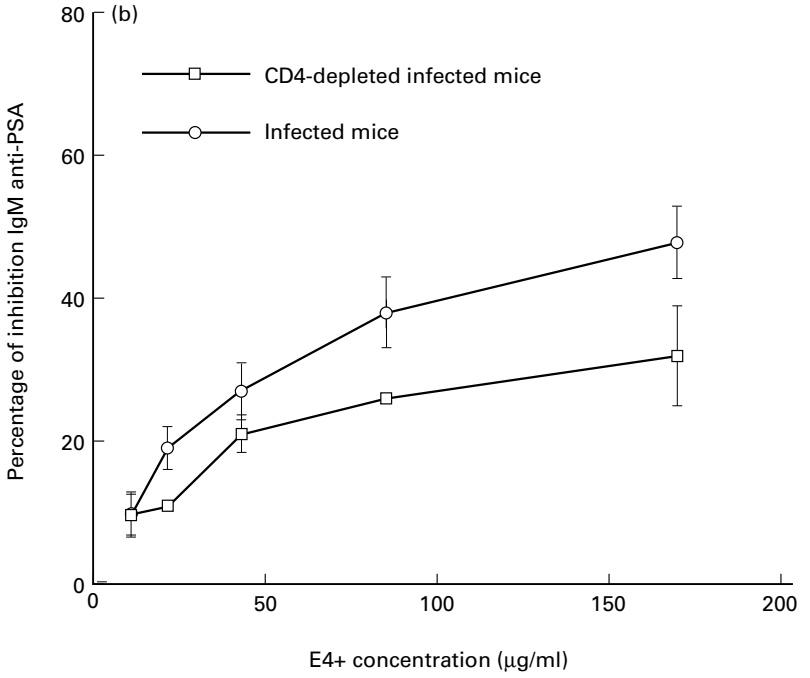


Figure 7 Inhibition of the reaction between anti-PSA antibodies and PSA using the fraction $E4^+$ as inhibitor. The ability of $E4^+$ to be recognized by antibodies produced by CD4-depleted mice during immunization (7a) or infection (7b) was analysed by inhibition ELISA. Sera were incubated with different concentrations of $E4^+$ for one h at 37°C before being added to PSA-sensitized ELISA plates. Inhibition of IgM or IgG antibodies were analysed independently as described in Materials and Methods. The percentage inhibition was calculated for each concentration of $E4^+$ according to the equation:
Percentage inhibition $= [(OD_0 - OD_i)]/OD_0 \times 100$
OD_i: OD samples with inhibitor
OD_0: OD samples without inhibitor

DISCUSSION

In this study we have depleted BALB/c mice of $CD4^+$-cells to evaluate the production of specific antibodies to *Echinococcus granulosus* protoscoleces by T-independent mechanisms.

The first aim of this work was to investigate the existence of T-independent antigens in *E. granulosus* protoscoleces. Production of specific antibodies in CD4-depleted mice immunized with protoscolex somatic antigens (PSA) was investigated. Consistent with the expected features of a T-independent response, recognition of peptidic epitopes, immunological memory and isotype switching to IgG were dramatically inhibited in CD4-depleted immunized mice compared with control immunized mice (Figures 1 and 2). However, a significant specific IgM and IgG response was detected in CD4-depleted immunized mice. These results demonstrate that protoscoleces contain antigens that stimulate secretion of specific antibodies without the involvement of $CD4^+$-cells. Such T-independent stimulation contributed to the antibody response mainly after priming, but it seemed to be quantitatively less important in secondary response. This could be due to the expansion of memory cells in the secondary response, which is consistent with the significant increase of the specific IgG/IgM ratio from primary to secondary responses observed only in control immunized mice. However, though not quantitatively relevant, T-independent stimulation may be qualitatively important in secondary response. This is suggested by results from Western blot analyses where the recognition pattern of carbohydrate epitopes by CD4-depleted immunized and control immunized mice sera were similar. Therefore, the production of antibodies specific to carbohydrate epitopes in mice immunized with PSA may be mainly T-independent.

The second aim of this work was to evaluate the relative contribution of a T-independent stimulation to the overall antibody response in early infection. Therefore, in a second experiment we analysed the immune response in both normal and CD4-depleted mice infected with *E. granulosus* over a period of seven weeks. Comparison of the antibody responses of the two experimental groups suggested that the production of specific IgM in early infection was mainly T-independent but the production of specific IgG was T-dependent (Figure 3).

It is well described that T-independent (TI) antigens can be classified into two categories: type-1 and type-2 TI antigens (Mond *et al*. 1995). Type-1 TI antigens are those that induce polyclonal activation and secretion of IgM antibodies. Type-2 TI antigens do not behave as polyclonal activators and can induce switching to IgG3 in mice. The observations that both CD4-depleted and control infected mice produced similar titres of specific IgG3 suggest that IgG3 during infection was induced by type-2 T-independent antigens (Figure 5). Apart from these observations, the elevated titres of IgG1 produced by control infected mice indirectly point to a quantitative relevance of T-dependent over T-independent responses in the presence of $CD4^+$-cells. In support of this idea, Western blots results suggest that recognition of carbohydrate epitopes may not be qualitatively predominant in early infection (Figure 4).

To carry out a preliminary isolation of protoscolex T-independent antigens by affinity chromatography, we used the monoclonal antibody E492/G1 specific for a carbohydrate epitope highly expressed in PSC. The epitope recognized by E492/G1 contains the motif Galα(1,4)Gal. This moiety is present in the P1 molecule so the P1 blood group reactivity described in HCFA (McManus & Bryant 1995) may be present in the antigen to which E492/G1 binds. Our results showing that E492/G1 recognized antigens from cyst wall and protoscoleces is in accordance with the presence of P1 activity in protoscolex tegument (Makni *et al*. 1992) and cyst wall (Russi *et al.* 1974; Dennis *et al*. 1993). Proliferation assays and the fact that antibodies from CD4-depleted mice recognized $E4^+$ (Figure 7) suggest that this fraction may contain type-1 and type-2 T-independent protoscolex antigens.

Collectively, our results suggest that *E. granulosus* protoscoleces contain immunogenic T-independent antigens. During immunization the production of specific IgM and a great proportion of IgG in primary response may be entirely induced T-independently. In addition, the recognition of carbohydrate epitopes in secondary response seems to be independent of $CD4^+$-cells. In early infection, specific IgM and IgG3 antibodies would be stimulated by T-independent antigens, although this response may be quantitatively less relevant than T-dependent stimulation. Finally, we used a monoclonal antibody to purifiy a fraction of protoscoleces which may contain putative type-1 and type-2 T-independent antigens. Isolation and biochemical characterization of type-1 and type-2 T-independent antigens from this fraction will be the aims of future work.

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Abbreviations: AU: arbitrary units; BSA: bovine serum albumin; CFA: Complete Freund's Adjuvant; E/S: excretion/secretion antigens; FCS: foetal calf serum; FITC: fluorescein

isothiocyanate; HCFA: hydatid cyst fluid antigens; HCWA: hydatid cyst wall antigens; HSA: human serum albumin; ip: intraperitoneally; iv: intravenously; MW: molecular weight; PSA: Protoscolex somatic antigens; PSC: protoscoleces; TI: T-independent; SI: stimulation index; SMA: specific mitogenic activity.

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