Intranasal immunization of mice with *Echinococcus granulosus* surface antigens Iscoms evokes a strong immune response, biased towards glucidic epitopes

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SUMMARY

The present work describes the preparation and characterization of iscoms from tegumental antigens of Echinococcus granulosus *protoscoleces, and their use as immunogens in mice by intranasal and subcutaneous routes. Iscoms given at 10 mg doses evoke a significant antibody response when administered i.n. and a strong and long lasting antibody response by s.c. route. The Ag administered i.n. in a non-adjuvanted form as a booster was not immunogenic. The i.n. route of immunization induced higher IgA serum titre in relation to IgG than the s.c. route and antisera of slightly higher avidity. Also, ten fold lower IgG2a/IgG1 and ten fold higher IgG3/IgG1 ratios were observed for the i.n. protocol compared to the s.c. one. Moreover, the mucosal route of immunization induced more efficiently antibodies of both isotypes directed to carbohydrate epitopes. The differences between immune response generated by i.n. and parenteral immunizations should be taken into consideration, particularly in those cases in which protective antigens are glycosylated.*

Keywords *Echinococcus, glycoconjugates, intranasal, Iscoms, MALT*

INTRODUCTION

With the advances of microbiology and immunology, essential components for induction of protective immunity against a wide variety of pathogens, i.e. protective antigens, have been identified. However, the limited success faced by vaccines against parasites is not only frustrating, but calls for a much more stringent and innovative search for the mechanisms that should be triggered by immunization to achieve protection. To render the protective antigens sufficiently immunogenic appropriate adjuvants or adjuvant formulations are required encompassing immunological optimal presentation, i.e. in several copies in a submicroscopic particle, such as a multimer (micelle or proteasome), a liposome (Morein & Simons 1985), or an immuno stimulating complex (iscom) (Morein *et al.* 1984). An inadequate immune response is not only irrelevant but may even favor the invading organism (Heinzel, Sadick, Mutha *et al.* 1991), thus induction of a proper response is a relevant research goal. A similar situation is faced with other pathogens in which the protective antigens are glycoconjugates.

Iscoms have been prepared with a variety of Ag, from many different pathogens (Morein *et al.* 1987). In all systems tested the immunogenicity was greatly enhanced in terms of antibody as well as cell mediated immunity, encompassing cytotoxic T-cells and T helper cell responses (Morein & Höglund 1990, Jones *et al.* 1988, Takahashi *et al.* 1990, Mowat *et al.* 1991, Villacres-Eriksson, M, PhD. thesis, Uppsala, 1993). Furthermore, the dose of Ag required to elicit a strong immune response is considerable reduced when presented in iscoms. For a number of pathogens protective immunity has also been achieved by immunization with iscoms (reviewed by Morein & Åkerblom 1990). Iscoms have also been used to deliver Ag by oral (Mowat *et al.* 1991, Mowat & Donachie 1991) or i.n. routes (Jones *et al.* 1988, Lövgren 1988) implicating that local mucosal

inductive sites are accessible for them. Very few efficient protective immunogens have been prepared against helminth parasites (reviewed by Wilson 1993), therefore, it is most important to test the potential of iscoms for immunization with helminth antigens, and particularly to analyse the potential use of these iscoms for mucosal stimulation and their capacity to affect the Th1/Th2 balance.

Echinococcus granulosus is a helminth parasite which causes a zoonotic disease known as echinococcosis/hydatidosis. The larval stage (hydatid cyst) infects both livestock herbivores and humans whereas the adult lives in the intestine of carnivores (mainly canines) and the eggs are spread when passed with host faeces. Within the fertile cysts are the protoscoleces (PSC), derived from the germinal layer, which, if eaten by a dog, evaginate and develop in the small intestine. When ingested by herbivores or humans, the eggs hatch larvae (oncospheres) that invade through the intestinal wall and evolve to hydatid cysts (reviewed by Heath 1986 and Thompson 1986). Both developmental stages interact with the immune system at mucosal level, for the entire infection period (up to two years) for the definitive host and at least during the early phases of infection for the intermediate host. Protective Ag have not been described yet for echinococcosis/hydatidosis but numerous efforts are being conducted in their search (Osborn & Heath 1982, Dempster *et al*. 1991, 1992, Hernández & Nieto 1994), mainly by use of experimental infection models in mice. Up to 70% protection against secondary infection in mice was reported (Hernández & Nieto 1994) by immunization with a preparation from PSC tegumental antigens. In addition, Ferragut & Nieto (1996) have reported that glycosilated Ags from *Echinococcus granulosus* are highly immunogenic during secondary infection and Miguez, Baz & Nieto (1996) reported that carbohydrates from the tegument of PSC may be involved in immune evasion. Research on the significance of the route of immunization to the outcome of an antibody response to peptidic or glucidic tegumental epitopes of *Echinococcus granulosus* PSC is relevant.

The present work describes the preparation and characterization of iscoms containing surface molecules of *E. granulosus* PSC, and the analysis of their immunogenicity in mouse when administered either by a systemic route (s.c.) or by two different mucosal routes (i.n. and p.o.).

MATERIALS AND METHODS

Antigens

Tegumental Ag were extracted from viable PSC (obtained as described by Carol *et al*. 1989) by solubilization in PBS containing different decanoyl-N-methylglucamide (Mega

10, Sigma, USA) concentrations ranging from 0·01% to 1·5%. One volume of decanted PSC was incubated with two volumes of buffer containing Mega 10 for two h in an end-over-end rotator. Supernatant was spun at 4000 rpm for 15 min on a bench centrifuge. After the extraction PSC were observed under light microscope to verify their integrity. This PSC tegumental extracted Ag (PSEx) was used for iscom preparation.

Somatic PSC soluble Ag was obtained by ultrasound disruption of 1 ml of viable PSC in 10 ml of PBS containing 5 mM EDTA and 2 mM phenylmethylsulphonyl fluoride. Ten sonication cycles of 15 s each every min were applied using half of maximum power with a Blackwell ultrasound disrupter, and subsequently cleared by centrifugation (15 min at $5000 \times g$).

Total PSC Ag was obtained by incubating viable PSC either with 2% SDS in water for one h at 50°C and freeze/thawing ($-70°C/37°C$) or directly by treating PSC with resorcinol sulphuric acid reagent (see carbohydrate analysis).

Iscom preparation and characterization

PSC iscoms were prepared according to Lövgren & Morein (1988). PSEx was ultracentrifuged over two layers of 10% sucrose in 0·1 M NaCl, 50 mM Trix pH 7·5 (TBS) with 0·2% Mega 10 and 30% sucrose in TBS respectively for two h at 39 000 rpm on a 41·14 swing out rotor (Kontron, Sweden) at 18°C. The top layer fraction was collected and Quil A (Spikoside, Iscotec, Sweden) added to a final concentration of 0·1% and 25 mg/ml of a 1:1 (w/w) mixture of cholesterol and L-a-phosphatidylcholine (Sigma). This preparation was thoroughly dialysed against TBS for 48 h, and spun again over stacked sucrose layers (5% sucrose, 0·2% Triton X-100 in TBS, 10% sucrose in TBS and 20% sucrose in TBS) for 18 h at 39 000 rpm and 10°C. After this centrifugation the supernatant was separated in the 70% upper fraction (f1), and the 30% lower fraction (f2). The pelleted iscoms were resuspended in PBS. Iscoms were also prepared from PSEx following the original dialysis protocol (Morein *et al.* 1984) in order to compare performances and antigen incorporation. Morphology was controlled by means of electron microscopy (negative staining with ammonium molibdate). Protein content analysis (Bradford test and SDS-PAGE), ELISA (Carol *et al.* 1989) and Dot-blotting (Harlow & Lane 1988) were performed on the fractions of analytical ultracentifugation to verify that iscoms structures and proteins co-migrated in the gradient and maintained immunogenicity. Antibody recognition patterns were analysed by immunoblotting and carbohydrate composition analysed by assessing the reactivity of the resolved glycoprotein bands with different lectins (see lectin blotting).

ELISA

For antibody quantification indirect ELISAs were used with PSEx (15 μg/ml solution) or iscoms (2 μg/ml suspension) adsorbed in PBS on ELISA polystyrene plates (Maxisorp, Nunc, Denmark) (following the conditions described by Hernández *et al*. 1994). A pool of PSEx immunized mouse sera was used as Ab standard reference (mAbS) and Arbitrary Units (AU) were assigned to this Ab reference for each isotype (12·800 AU/Ml of IgG, 3·200 AU/mol of IgM and IgA) so that isotype titres correlate (same titre for OD600 = 0·5), in order to allow for isotype comparison. AU and OD600 data corresponding to dilutions of the standard reference were correlated by linear regression and minimum squares fitting curves were obtained. ELISA data corresponding to each sample were converted to AU equivalent to the respective reference using these curves, for analytical consistency (Malvano *et al.* 1982). These values were multiplied by the dilution factor and all the values of a same sample were used to calculate the arithmetic mean, standard deviation and variation coefficient.

Specific Ab of all IgG subclasses were detected using goat specific unlabelled antisera (Nordic, The Netherlands) and anti-goat PO conjugate (Sigma) in subsequent steps. These results were expressed as titre ratios related to corresponding IgG1 titres.

For IgA and IgM determinations the sera were depleted of IgG using a Protein G Sepharose 4FF (Pharmacia, Uppsala, Sweden) column with an estimated ten fold excess capacity. Five ml 2 N Tris pH 8·0 were added to 20 ml of serum sample, and passed through the column followed by PBS pH 7·8. The non-bound fractions were collected, diluted as required to correlate to the simulation sample and checked to be free of IgG. The column was reconstituted after each cycle by washings with 2 ml of 2 M Urea, 2 ml 1 M LiCl and 3 ml 0·1 M Citric Acid pH 3·0. The dilution factor of the non-bound fraction was calculated using OD readings at 260 nm of a simulation sample (2 mg/ml dextran blue in 1 M LiCl) before and after passage through the column, exactly in the same way as a serum sample.

Mean IgG avidities were compared between sera from lots A and B exhibiting maximum secondary response titres (20 days after booster). An ELISA method including KSCN elution (Pullen, Fitzgerald & Hosking 1986), was used and the Avidity Index calculated as the KSCN concentration (in moles/l) that eluted 50% of the originally bound Ab (by interpolation of the KSCN concentrations immediately above and below 50% AU/ml).

Antibody recognition of glycosidic moieties for each isotype was evaluated by ELISA (Woodward, Young & Bloodgood 1985). Briefly, half of a plate coated with native Ag was incubated with 100 ml/well of 20 mM NaIO4 in 50 mM sodium acetate buffer pH 4·5, one h in the dark, and the other half was treated with acetate buffer only. Afterwards all wells were incubated for 30 min with NaBH4 50 mM in PBS. After washings, all wells were processed as for the regular ELISA. Results were expressed as the percentage of O.D. reduction exhibited by the NaIO4 treated Ag well related to the non-treated Ag well for each sample.

Ag characterization

Protein content of the Ag fractions was determined, depending on the different interferences, either by the Bradford method, the bicynchoninic acid method (BCA, Pierce, USA) or by the Lowry method (for whole intact PSC) with bovine serum albumin always as standard. Protein composition of Ag was analysed by SDS-PAGE under reduced conditions (Laemmli, 1970).

Carbohydrate content of the Ag was measured by a modified orcinol-sulfuric assay (White & Kennedy 1986). Sample (200 ml) and reagent (800 ml 2 mg/ml resorcinol, Sigma, in concentrated sulphuric acid) were mixed, heated at 80°C for 15 min. and cooled to 20°C. Absorbances were read at 420 nm, and referred to a standard curve of Dextran Sulphate (T2000, Pharmacia, Sweden).

Lectin blotting was performed on nitrocellulose membranes (0·45 μm nominal pore, Schleicher & Schuell, Germany), as described (Hammar, Eriksson & Morein 1989), with a battery of biotinylated lectins (Vector, USA) diluted according to manufacturers instructions in 0·15 M NaCl, 20 mM Tris, 0·1 mM CaCl2, 0·1 mM MnCl2, 1·0% BSA pH 7·5 (LBB). After washings (three times with LBB, 10 min), they were incubated with an alkaline phosphatase (AP) ABC complex (Vector) in LBB following the manufacturers' instructions. After washings, strips were developed as for the immunoblotting.

Immunoblotting was performed with 48 mM Tris base, 39 mM glycine, 0·037% SDS and 20% methanol as transfer buffer (Harlow & Lane 1988) in a semidry transfer apparatus (LKB, Sweden). Nitrocellulose was blocked by incubation with PBS 5% skimmed milk for one h and strips were then incubated with the Ab appropriately diluted in PBS-T 1% skimmed milk, washed with PBS-T (three times 10 min) and incubated with the respective AP conjugated anti IgG antibody (BioRad) in PBS-T 1% skimmed milk, washed again and developed with a solution of nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) in 0·1 M carbonate buffer, 10 mM MgCl$_2$, pH 9·8 (BioRad).

For dot blotting Ag were applied by vacuum to the nitrocellulose with a Bio Dot apparatus (BioRad, USA) in concentrations ranging from 400 to 3·1 ng/dot. The membranes were blocked as for the immunoblot.

Mice

CD1 mice obtained from Charles River Breeding Laboratory, Inc. (Willmington, MA, USA) and thereafter bred at our own animal facility were used after reaching 25 g of body weight in sex and age matched lots.

Immunization

Mice (six animals per lot) were immunized on days 0 and 29 with 10 µg doses of iscoms subcutaneously (Lot A), intra-nasally (Lot B), perorally (Lot C), PSEx perorally (Lot D) and immunization buffer perorally (Lot E). All Ags were diluted in 3% sodium bicarbonate buffer pH 9·0, 1% gelatin (immunization buffer). Additional control lots (i.n. administration of buffer only and PSEx) were included in a second experiment. For i.n. administration of Ag mice were put under light ether anaesthesia. Blood was collected periodically at 5–7 day intervals from all individuals according to the guidelines in the First Report of the BVA/FRAME/

RSPCA/UFAW (1993). Sera were separated and stored at −20°C until tested.

Systemic antibody responses were followed by ELISA of pooled sera. Intra-group variation was assayed on selected samples (days 0 and 53) by testing all individual sera and Percent Variation Coefficient was calculated ($100 \times SD/$ mean value).

RESULTS

The optimum concentration of detergent (Mega 10) for extraction of surface PSC Ag is 1% (data not shown). Under these conditions PSC tegumental Ag were solubilized with only a minor proportion of disrupted PSC observed by microscopic examination, and little debris was produced.

Four different extractions were performed under these conditions yielding $2 \cdot 1 \pm 0 \cdot 1$ mg of soluble proteins from 2·0 ml of decanted PSC. The carbohydrate/protein ratio (CH:P) in w/w of total PSC Ag was 1·7. Corresponding ratios exhibited by the other Ags were 1·0 in the case of somatic PSC Ag, 2·0 in the case of insoluble material after ultrasound disruption and 1·9 in the case of PSEx.
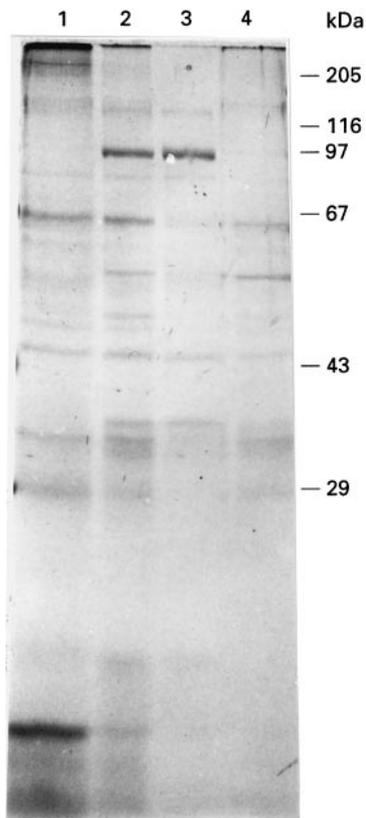

Figure 1 Antigen composition of protoescoleces extract (PSEx) in Iscoms (1), PSEx (2), f2[1] (3) and f1[1] (4) as seen after SDS-PAGE (12%) separation under reducing conditions. [1] f1 and f2 are gradient fractions obtained by ultracentrifugation (see Materials and Methods). Iscoms are recovered in the pellet.
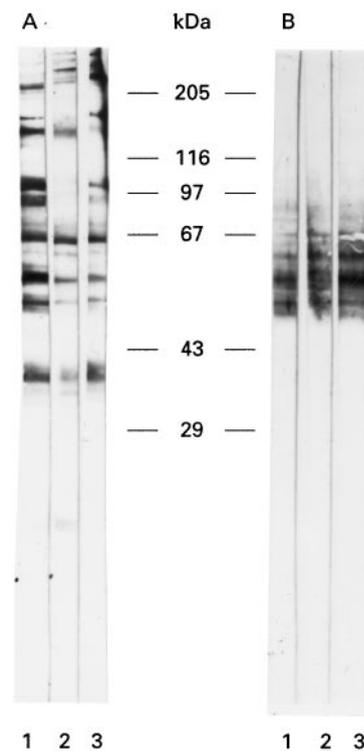

Figure 2 Immunoblott analysis of antigen patterns of protoescoleces extract (PSEx) (1), PSEx Iscoms (2) and f2[1] (3) after 12% SDS-PAGE separation under reducing conditions detected with a Human positive reference serum (A) and a Mouse antibody reference (MoAbs-II) (B). [1]See Figure 1.

Both the preparative methods yielded iscoms with the previously reported (Morein & Höglund 1990) cage like, spherical shape and size (35–45 nm) as seen by electron microscopy (data not shown). The protein contents of the iscoms suspensions (0·7–0·9 mg/ml) correspond to circa 30% recovery from the total protein content of the starting material (PSEx). The percentages of non-incorporated Ag distributed in the two fractions of supernatants (from the last centrifugation) were 25% in f1 and 45% in f2. SDS-PAGE patterns of PSEx, iscoms, and the non-incorporated proteins in f1 and f2 are shown in Figure 1. There is a selective enrichment of the low MW Ag in iscoms, as well as of the components above 200 kDa, and a depletion of the 120, 97 50 and 35 kDa Ag although most of the Ag of PSEx are represented in the iscoms. This is confirmed in Figure 2, where Ag composition of PSEx, f2 and iscoms
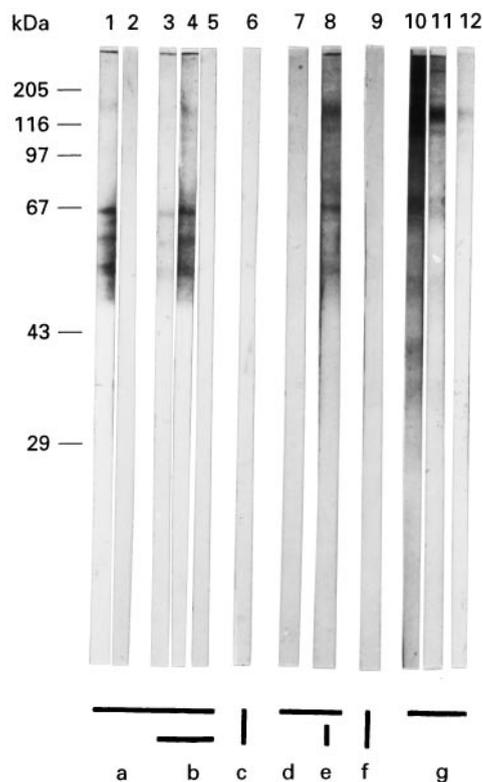

Figure 3 Carbohydrate analysis by lectin blot of glycoproteins incorporated into iscoms. Carbohydrate moieties (a) N-Acetil-Galactosamine; (b) Galactose; (c) Galactosyl Beta-1.3-N-Acetil-Galactose; (d) N-Acetil-Glucosamine; (e) Sialic Acid; (f) Fucose [alfa linked]; (g) Mannose [alfa linked] present in the Ag incorporated onto PSEx Iscoms after SDS-PAGE (12%) and transference to nitrocellulose membranes detected by lectins from Soybean (1), *Dolichus biflorus* (2), *Ricinus comunis* (3), *Bandeira simplicifolia* (4), *Sophora japonicum* (5), Peanut (6), Wheat germ (succinilated) (7), Wheat germ (8), *Ulex europeaus* (9), Concanavalin A (10), *Lens culinaria* (11) and *Pissum sativum* (12).

according to immunoblotting patterns of the human and mouse (mAbS-II) positive sera are shown.

Lectin blotting patterns corresponding to carbohydrate moieties of Ag incorporated in iscoms are shown in Figure 3. One group of several Ag of relative MW in the range 48 to 68 kDa mainly reacts with galactose and N-acetylgalactosamine binding lectins while another in the range 110 to 200 kDa mainly reacts with mannose binding lectins. Sialic acid seems to be present in most of these glycosilated Ag.

Mice, which receive iscoms s.c., mounted an early, strong and long lasting systemic IgG response (Figure 4). IgM peaked at day 11 and 8 after priming and booster respectively. IgG increased steadily until day 24 post booster reaching 250 and almost 10^4 fold increases for primary and secondary responses, respectively; by day 70 post booster IgG titres had only decreased around 25% with respect to the maximum (47600 ± 2900 to 34900 ± 2100). IgA titres rose 1·5 times above background by day 21 after the priming and almost seven fold at day 39 post booster. Iscoms administered i.n. (10 mg/dose) induced systemic IgM and IgA specific responses of similar magnitude to those administered s.c. The i.n. route induced IgG response only about 4% of the latter (Figure 4). Individual variations of serum antibody responses, expressed as Variation Coefficient, for lot A (s.c.) were 56% (IgM), 25% (IgG) and 32% (IgA) while for lot B (i.n.) were 82% (IgM), 164% (IgG) and 53% (IgA) measured on day 53.

Control mice administered immunization buffer i.n. ($n = 5$) had no significant change in Ab titres after priming and booster. Baseline values (mean ± SD, Figure 4) from 4 extractions of all individuals were 45·5 ± 4·4 for IgM, 34·2 ± 5·1 for IgA and 14·6 ± 3·2 for IgG. Both iscoms and PSEx given by the p.o. route at 10 mg/dose failed to induce a systemic antibody response. Soluble PSEx (no adjuvant added) was not capable of inducing a systemic immune response at the same doses used for Iscoms, neither when given i.n. as a priming ($n = 5$) nor as a booster to animals already primed by another route ($n = 3$ for s.c. and $n = 3$ for i.n.).

Relevant qualitative differences were recorded, for two additional parameters assessed, between the two immunization routes. With regard to the proportion of antibodies recognising periodate sensitive epitopes, the s.c. route induced 55% of the IgM, 20% of the IgG and 45% of the IgA directed to periodate sensitive epitopes while the i.n. route induced 20% IgM, 45% IgG and 100% IgA against these epitopes (Figure 5) at day 53. The IgG subclass composition of the maximum secondary response showed significant differences for the two routes, particularly for IgG2a and IgG3 (Table 1). While s.c. immunization generated 56% of IgG2a and 1% of IgG3 with respect to the level
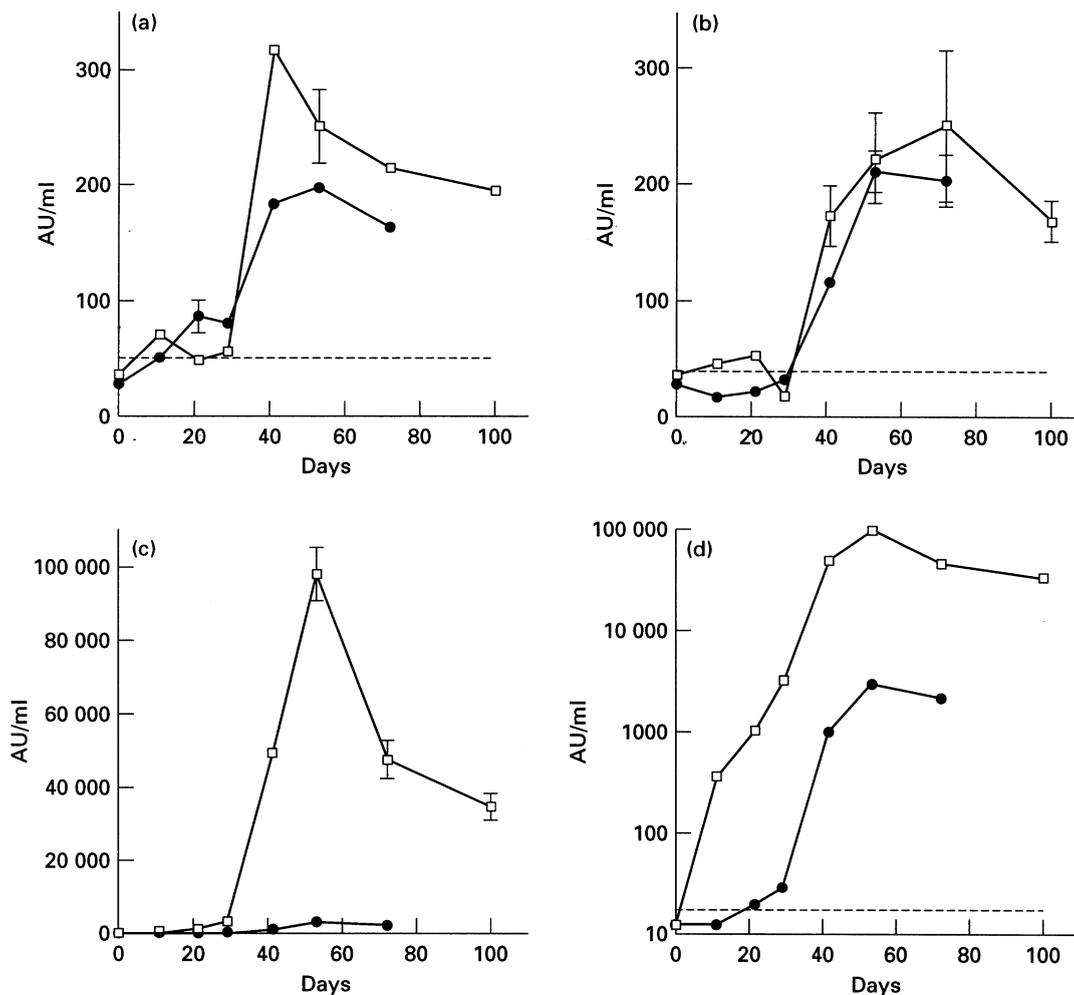
Figure 4 Serum antibody production kinetics measured by ELISA against protoescoleces extract (PSEx) of pooled samples from s.c. immunized mice (lot A; open squares) and i.n. immunized mice (lot B; closed circles). Panel (a): IgMs; Panel (b): IgAs and Panels (c) and (d): IgGs (linear and logarithmic scales). Dashed lines are mean + SD of four extractions from five mice immunized i.n. with buffer only. On days 0 and 29 mice were immunized with PSEx Iscoms. Vertical bars represent error for each determination.

of IgG1, i.n. immunization induced 6% IgG2a and 9% IgG3 with respect to IgG1.

High Avidity Indexes of IgG were observed both after the s.c. and i.n. immunization (2·1 and 2·5 respectively) on day 24 post booster, when highest titres were reached.

DISCUSSION

The relevance of the route of immunization to the outcome of the immune response is underlined in this study. First, we report that serum IgA and IgG subclasses profiles induced by immunization with *E. granulosus* iscoms are clearly different between mucosal and parenteral routes (in agreement with data reported by Lövgren 1988, with influenza virus iscoms), and deserve further studies. Second; analysis

of Ab reactivity with periodate sensitive epitopes also shows a difference in the immunogenicity of these epitopes, depending on the mode of Ag delivery. Both differences in antigen presenting cells available, as well as the lymphocyte repertoire and/or cytokine production locally at the administration site, may account for these phenomena. Although the biased Ab response against glycoconjugates obtained by a mucosal route of administration could be regarded as not promising for protection, its biological significance should be evaluated, since it differs qualitatively from that obtained after s.c. immunization. Confirmation in larger samples (analysed individually) both through IgG subclasses production and cytokine profiles should be carried out to establish if there are differences in Th1/Th2 balance between the two routes of immunization. As in other
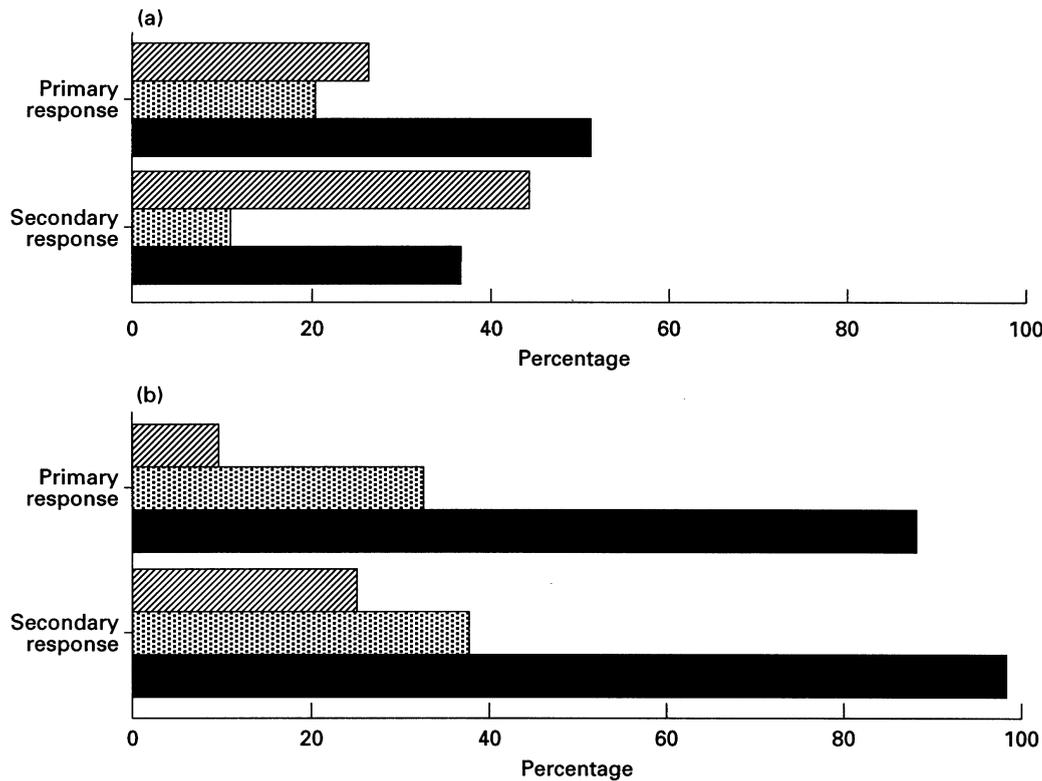
Figure 5 Proportion (in percentages) of carbohydrate containing epitopes[1] recognized by the Antibody response of the different isotypes (IgM: slashed bars; IgG: grey bars; IgA: solid bars) in mice after primary and secondary immunization with PSEx Iscoms by s.c. route (a) or i.n. route (b). [1]Carbohydrate moieties were oxidized by exposure to sodium periodate (see Materials and Methods). ▨ IgM; ▧ IgG; ■ IgA.

Table 1 Relative composition of IgG subclasses in serum following two s.c. or i.n. immunizations of mice

	Lot A[2]	Lot B[3]
Titre	95500	3600
IgG1/IgG1	1·0	1·0
Titre	53700	210
IgG2a/IgG1	0·56	0·06
Titre	17800	510
IgG2b/IgG1	0·19	0·14
Titre	1100	330
IgG3/IgG1	0·01	0·09

[1] Values are expressed as subclass titre and titre ratio to IgG1 for highest secondary response antibody concentrations (day 20 post booster) detected by ELISA with PSEx on the solid phase. [2] Mice in Lot A received PSEx Iscoms s.c. on days 0 and 29. [3] Mice in Lot B received PSEx Iscoms i.n. on days 0 and 29.

long term parasitic infections, there is non-protective Ab production (Th2 type) against hydatid infection in mice, while a Th1 type immunity could be related to protection (A. Baz and S. Dematteis, personal communication). Modulation of this balance could generate a hostile environment for establishment of the parasite and may be crucial for immunoprotection. Furthermore, Ab against glycoconjugates might be of limited use in intermediate hosts, but their protective role at mucosal surfaces, in definitive hosts, remains to be assessed.

We should also stress the fact that this bias towards carbohydrate moieties could be of use in the generation of Ab dependent immunity against glycoconjugates of other pathogens, as is the goal of conjugated vaccines.

Various concentrations of detergent do not seem to produce qualitative differences of the antigen composition in the extract of surface antigens from PSC. Up to 1% of Mega 10 there was a quantitative increase of extracted Ag, however, higher concentrations produce a different Ag pattern with additional bands, most probably due to the solubilization of some somatic Ag as well. The iscoms obtained from PSEx are morphologically and physochemically comparable to other reported iscoms. A

wide range of Ag were incorporated, although there was also a number of proteins not incorporated. We did not specifically assess the incorporation of the Ag of 187, 184, 148, 141 and 134 kDa described by Hernandez *et al.* (1994) as involved in protection, in the iscoms. The importance of the non-incorporated Ag (putatively with no hydrophobic domains) on the generation of protective immunity has to be assessed and, if necessary, a procedure to incorporate them into the Iscom structure should be followed (Reid 1992). Immunoreactivity analysis of the different fractions (ELISA, dot blot and immunoblot data) demonstrate that many of the Ag recognized by positive human and mouse reference sera are present in PSEx iscoms and retain their immunogenicity. ELISA readings obtained with iscoms and f2 both with the human and mouse control samples were lower than those for PSEx but titre ratios (positive/negative) were similar for all of these preparations.

The high CH:P ratios exhibited in the different Ag preparations suggest that parasite carbohydrates are strongly expressed at tegumental surfaces and consequently a surface extracted Ag would be enriched in these Ag. In fact, we found almost a two fold increase of glycoconjugates in the detergent extracted Ag. Carbohydrates also seem to be preferential targets for the immune system, as could be expected due to the abundant PSC glycocalix (Smyth & McManus 1989). This can well be, as is the case in other helminths, a parasite strategy for evasion (Dunne 1990). These findings are consistent with those reported by Miguez *et al.* (1996) and by Hernández *et al.* (1994). Figure 2 shows that a wide range of the incorporated Ags are in fact glycoconjugates and that galactose, N-acetilgalactosamine, mannose and sialic acid are the predominant moieties detected. The unexpected abundance of sialic acid could point to the incorporation of this molecule from the host. By means of being recognised as 'self', sialic acid could be critical for evasion of immunity not related to antibodies since inhibition of specific antibody reaction with this glycoside is not significant (Miguez *et al.* 1996).

We have considered periodate sensitive epitopes as glycosidic in order to assess the percentage of Ab directed to glycosidic epitopes, but it should be stressed that oxidation of the cis-diol containing carbohydrate moieties (Woodward *et al.* 1985) does not necessarily abrogate Ab binding, and may eventually modify the spatial configuration of non-carbohydrate or bystander epitopes.

The PSEx iscoms proved to be highly immunogenic, as the case is in other systems tested, and a high effect/dose ratio was observed. Primary and secondary response titres were high and long lasting with only a 25% decrease with respect to maximum titre after 70 days from the booster (lot A). Both iscoms and PSEx given by the peroral route at 10 mg/dose failed to induce a detectable systemic Ab

response or any degree of memory. This was further confirmed by immunizing those mice intranasally with either of the immunogens. No secondary Ab response could be recorded in these animals, but rather a poor primary response (data not shown), when at all. Formulation of these Ag in iscoms is crucial since soluble PSEx alone was not immunogenic by i.n. route.

The amount of Ag per dose (10 mg) is a much lower dose than what is generally used for mucosal immunizations (mg doses for mice), but we intended to assess iscoms' immunogenicity at low doses, which is one of their greatest advantages. Mowat *et al.*, (1991) reported 100 mg of ovalbumin incorporated into iscoms to be the amount with the highest immunogenic effect, by gavage administration, as a single dose. On the other hand, the B-subunit of the cholera toxin in iscoms is immunogenic after one oral immunization with 10 mg doses (studies in progress). Factors like degradation, dilution of the immunogen as well as physical barriers in the gut may be influencing the amount of antigen that reaches inductive sites, and may be the causes of the differences between the effect seen for NALT and GALT. It should also be noticed that only systemic humoral response was monitored, which is reported to be mounted after intranasal immunization (Quiding-Järbrink *et al.* 1995), but not necessarily after i.g. immunization.

The very high IgG titres induced, with relatively high avidities, were expected and confirmed to cause lower readings in the IgA and IgM ELISAs by competition and blockage of epitopes, if not removed. The depletion of IgG from the samples was envisaged as an alternative to a specific isotype capture ELISA, and it gave accurate IgM and IgA values, in our hands.

The generation of protective immunity by this immunogen should be tested both in intermediate and definitive hosts. We are currently undertaking immunization experiments with *Echinococcus granulosus* iscoms in dogs in order to establish its effectiveness to generate an adequate immune response. Once protective Ag are defined, immunoprophylaxis of the definitive host will imply at least a priming by a route involving lymphocytes of mucosal inductive sites. The knowledge on Mucose Associated Lymphoid Tissue (MALT) now available may permit us to tailor make a vaccine to induce protective immunity against Echinococcus infection. Iscoms may be one of the vaccine candidates for this purpose.

ABBREVIATIONS

Ag: Antigen/s; GALT: Gut Associated Lymphoid Tissue; i.n.: intranasal/ly; MALT: Mucose Associated Lymphoid Tissue; NALT: Nasopharyngeal Associated Lymphoid Tissue; p.o.: peroral/ly; PCS: Protoscoleces; s.c.: subcutaneous/ly.

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