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IMMUNIZATION PROCEDURE-RELATED IMMUNOGLOBULIN LEVELS IN THE DEVELOPMENT OF ANTIBODIES AGAINST *CYSTICERCUS CELLULOSAE*

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ABSTRACT

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Various immunization procedures were investigated in an effort to improve the number of hybridomas producing antibodies against *Cysticercus cellulosae*. Ten groups of 5 BALB/c mice were subjected to different immunization procedures and were bled repeatedly over a period of 68 days. The samples of sera thus obtained were tested by enzyme linked immunosorbent assay: total immunoglobulins, IgG and IgM levels were determined. In general, total anticyst antibody titres increased during the course of immunization but in 3 groups the final titre was lower than the maximal antibody titre. Overall, immune tolerance did not appear to be a problem and longer immunization programs seemed to end with slightly higher antibody levels. So far, 4 mice from the group that exhibited the highest immunoglobulin levels have been used for hybridoma production. Out of 124 hybridomas thus obtained, only 1 secreted antibodies against *Cysticercus cellulosae*.

INTRODUCTION

One of the most common problems facing investigators embarking on the production of monoclonal antibodies is obtaining sufficient numbers of antibody-producing hybridomas (specific efficiency or SE). This is particularly true when soluble antigens are used (Siraganian, Fox & Berenstein, 1983; Stahli, Staehelein & Miggiano, 1983; Reading, 1986). While high numbers of hybrid cells may result from a fusion experiment, often few or none of these hybrids produce antibodies of the desired specificity. Furthermore, hybridomas may secrete antibodies which react specifically or non-specifically with bovine serum albumin (BSA) or even the plastics used in the antibody assay (Campbell, 1984).

It is generally, but not universally, accepted that a high level of circulating antibody favours the production of antibody-secreting hybridomas (Zola & Brooks, 1982; Campbell, 1984; Spitz, 1986). Antibody levels are influenced by the degree of immunization, both under-immunizing and over-immunizing having negative effects. Although some authors have studied 'conventional' immunization protocols related to the production of monoclonal antibodies (Zola & Brooks, 1982; Siraganian *et al.*, 1983; Stahli *et al.*, 1983, Campbell, 1984), the number of publications on this subject is surprisingly low compared with the mass of literature on hybridoma technology. We have undertaken the production of monoclonal antibodies against *Taenia* spp. and were faced with the problem of low SE. Consequently, we have investigated antibody levels of groups of mice subjected to different immunization protocols in the hope of developing an immunization procedure that would result in a high SE.

MATERIALS AND METHODS

The parasite antigen used in immunization and the immunoassay consisted of a 105 000 *g* soluble fraction of *Cysticercus cellulosae* prepared as previously described (Pammenter & Rossouw, 1984). Ten groups of 5 female BALB/c mice, 9 weeks old, were immunized according to different protocols, as illustrated in Fig. 1. The first injection was performed with Freund's complete adjuvant[1], while in subsequent injections incomplete adjuvant, mixed 1:1 with the antigen, was used. A total volume of 250 $\mu\ell$ (groups 501 and 5001) or 100 $\mu\ell$ (all other groups) was injected intra-peritoneally. These procedures were adapted from methods of Nowotny (1979) and Stahli *et al.*, 1983. Four groups of mice were immunized with 50 μg of cysticercus antigen per inoculation, and another 4 groups with 500 μg. A positive control was injected with 50 μg of BSA per injection, while the negative control group was not treated. Animals were bled at various intervals (Fig. 1) from the tail vein and after separation, sera were stored at −20 °C until required.

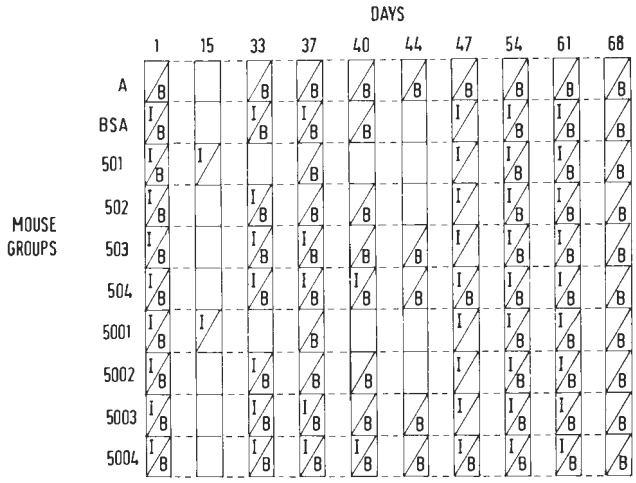

FIG. 1 Immunization and bleeding protocols

A, negative control not injected; BSA, control group injected with 50 μg per mouse; 501–504, groups of mice inoculated with 50 μg cyst antigen protein per mouse; 5001–5004, groups of mice inoculated with 500 μg cyst antigen protein; I, injection of antigen; B, bleeding of mice. Volumes of adjuvant are given in the text.

The enzyme linked immunosorbent assay (ELISA) was performed in 50 $\mu\ell$ volumes, using standard procedures (Douillard & Hoffman, 1983). Microtitre plates[2] were activated with 250 ng antigen per well and serum was assayed at a dilution of 1:160. This afforded best differentiation between control positive and negative samples. All samples from each group of mice were tested on the same ELISA plate to obviate inconsistencies in the plates. Negative samples were tested individually, 8 on each plate. Bound total antibody was detected with polyspecific horseradish peroxidase conjugated anti-mouse immunoglobulin[3]. Specific immunoglobulins

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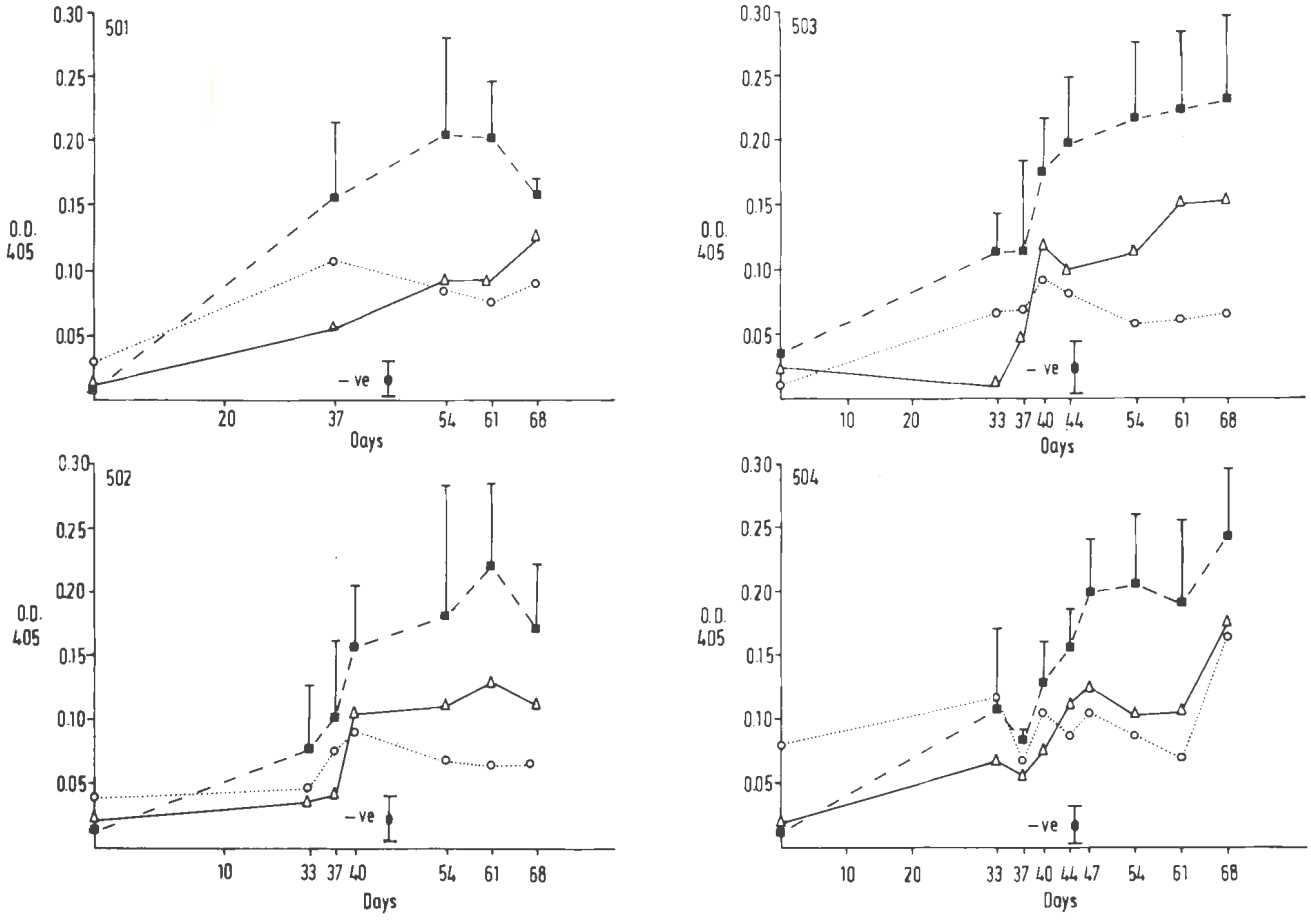

FIG. 2 ELISA results of sera from mice inoculated with 50 μg cyst antigen protein per injection

□ ---------- □ total anticyst Ig

△ —— △ anticyst IgG

○ ····· ○ anticyst IgM

(Ig) were detected with alkaline phosphatase conjugated anti-mouse IgG or IgM[4]. Colour was developed with o-phenylene diamine for horseradish peroxidase and p-nitrophenyl phosphate for alkaline phosphatase.

RESULTS

ELISA results of sera from mice immunized with *Cysticercus cellulosae* antigen are presented in Fig. 2 & 3. The antibody titres of the different classes of Ig can be compared qualitatively but, because of the different detection systems used, this could not be done quantitatively. In general, total anticyst antibody titres increased during the course of immunization, but in 3 experiments (501, 502 and 5001) the final titre was lower than the maximal antibody titre. While this was not statistically significant (P>0,05) it may represent the start of reduced antibody output.

In keeping with established data (Hood, Weissman, Wood & Wilson, 1984) specific IgM levels generally showed an early rise followed by a decline which was most marked in Experiment 5002. IgG, on the other hand, showed a general increace with repeated inoculations. Exceptions were 2 experiments (504 and 5003) in which the IgG and IgM levels appeared to remain parallel, both showing primary and secondary responses.

Mice immunized with BSA were tested for total anti-BSA antibodies only and these showed an increasing response with repeated inoculations (results not given).

[4] Sigma, USA

DISCUSSION

The level of total immunoglobulins at the end of the immunization protocol is an aspect which we consider to be of immense importance. In this respect, experiments involving the highest numbers of boosts (503, 504, 5003 and 5004) all end with the highest Ig titre and, furthermore, IgG was still on the increase at this stage. This suggests that, using these immunization procedures, immunological tolerance with concomitant decrease in Ig titres did not pose a problem. Shorter immunization schedules (501, 502 and 5001) end with a reduction in total anticyst antibody titre. When other immunization schedules (503, 504, 5003 and 5004) were used, a slight decrease in titre was also observed during the early stages of immunization. While these difference were not statistically significant (P>0,05) they could have been due to a decline in IgM anticyst antibody titres and the recovery due to an increase in IgG antibodies.

One disconcerting finding is that in 2 groups (504 and 5003) the IgG and IgM curves followed the same pattern throughout the immunization procedure with fluctuations in titre occurring simultaneously. This is not consistent with the generally accepted scheme whereby IgM peaks early during the immune response and is followed by high titres of IgG (Hood *et al.*, 1984). We are unable to explain this phenomenon; it is possible, albeit unlikely, that accidental inoculation with a contaminating cross-reacting antigen part way through the immunization schedule could have occurred. The possibility of these results being due to non-specific reactions with either BSA or the ELISA plates, although unlikely, cannot be ruled out.

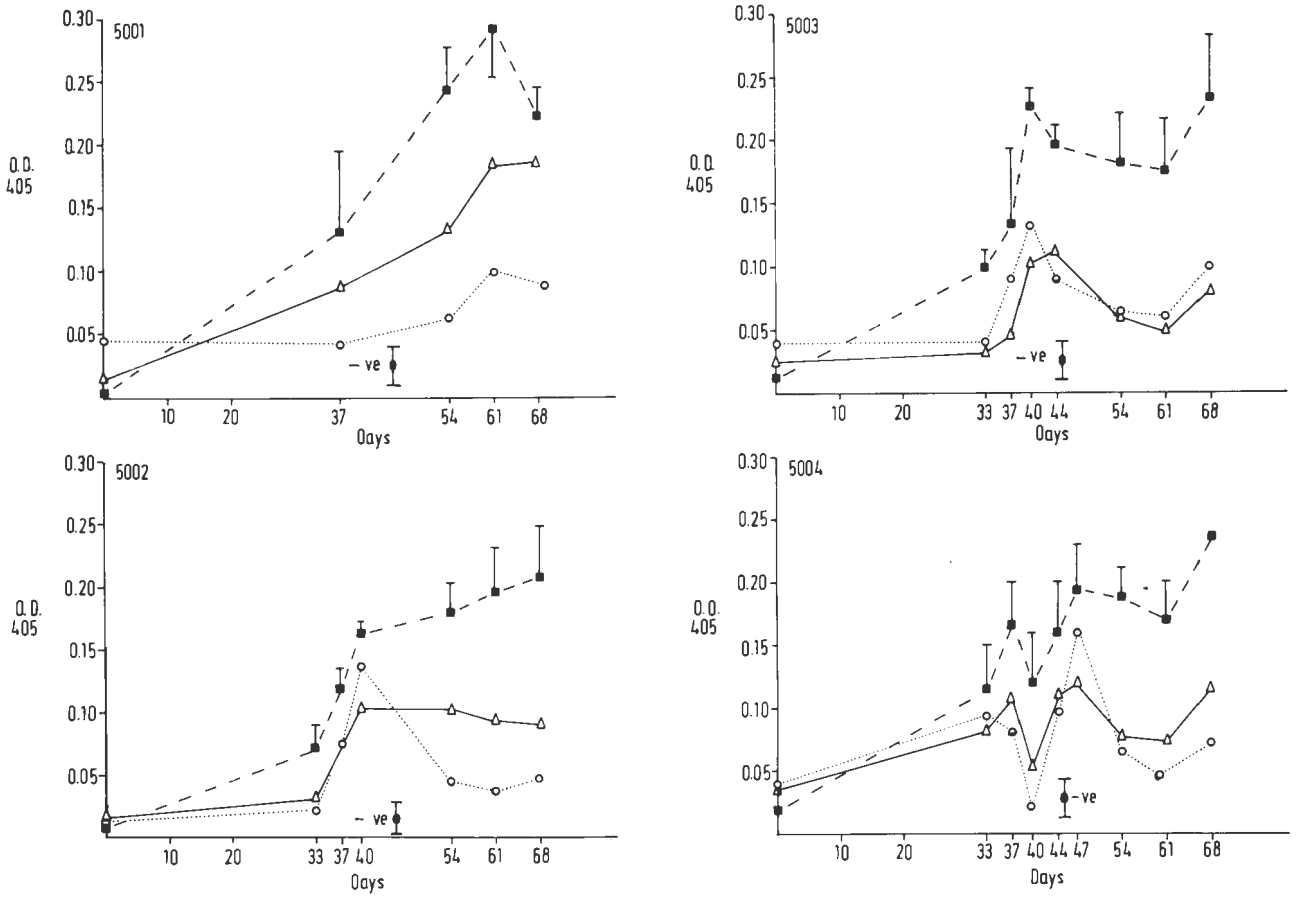

FIG. 3 ELISA results of sera from mice immunized with 500 μg cyst antigen protein per injection

□ --------- □ total anticyst Ig

△ ——— △ anticyst IgG

○ ····· ○ anticyst IgM

From our point of view, the most crucial factor is to determine which of the immunization procedures produced the highest IgG and total antibody titres against the antigen. Statistical analysis showed that the different protocols do not differ significantly from one another. This may be related to the variation observed within the group, since we found that some of the mice responded better to this antigen than others. Subjectively, it appears that 5001 was the group that showed the fastest and highest response, and since the closing of this study 4 of the mice from this group have been used for hybridizations at different times.

Mice were immunized once more 4 days prior to fusion, at which stage antibody titres had increased, thus confirming that immune tolerance was not a problem. That serum was positive for anticyst antibodies at dilutions between 1:640 and 1:1280 suggests that this protocol is acceptable in terms of hybridoma production. Despite this, spleens were small (more than 1×10^8 cells were recovered only from one animal) and only 1 out of 124 hybridomas obtained produced antibodies against *Cysticercus cellulosae*. This gives an SE of less than 1 %, which is comparable with the results of other studies in which soluble antigens were used (Lin & Chen, 1985; Lane, 1985; Danford, Campbell, Leef & Beaudoin, 1982). However, these SEs are low when compared to the up to 41 % achieved by Stahli *et al.* (1983) and up to 78 % by Cianfriglia, Mariani, Armellini, Massone, Lafata, Presentini & Antoni (1986), who also used soluble antigens.

Techniques designed to increase the relative number of antibody-producing lymphocytes have been described; these include separating spleen cells (Van Mourik & Zeijlemaker, 1986) and immunization directly into the spleen (Spitz, 1986), or by *in vitro* or *in vivo* culturing of prestimulated lymphocytes (Siraganian *et al.*, 1983). Alternatively, Stahli *et al.* (1983) recommended the use of antigen without adjuvant in the final boost before hybridization. Preliminary results suggest that this may be more effective in the case of cysticercus antigen. Other avenues will now have to be explored.

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