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Bovine Reissner's fiber (RF) and the central canal of the spinal cord: an immunocytochemical study using a set of monoclonal antibodies against the RF-glycoproteins

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Abstract. The subcommissural organ secretes *N*-linked complex-type glycoproteins into the cerebrospinal fluid. These glycoproteins condense to form Reissner's fiber (RF), which extends along the fourth ventricle and central canal of the spinal cord. A set of three monoclonal antibodies (Mabs 3E6, 3B1, and 2A5) has been obtained using these glycoproteins as immunogens. Competitive and sandwich enzyme-linked immunoassay methods have demonstrated that the three monoclonal antibodies are directed against different epitopes, and that there is no competition among them for their binding to glycoproteins of RF. Mab 3E6 displays immunoblotting properties that are similar to those of a polyclonal antibody against the pool of glycoproteins from RF, but that are different from those of Mabs 3B1 and 2A5. All three antibodies immunostain the bovine subcommissural organ and RF. A population of ependymal cells is stained by the polyclonal antibody, and Mabs 2A5 and 3E6, but not by Mab 3B1. The material present in a population of ependymal cells of the central canal, and the glycoproteins secreted by the subcommissural organ thus probably have partial chemical identity. Some evidence suggests that the immunoreactive ependymal cells are secretory cells. The luminal surface of the central canal is coated by a thin layer of material with immunocytochemical characteristics different from those of the ependymal cells; such a coat may correspond to material released from RF.

Key words: Subcommissural organ – Reissner's fiber – Central canal – Monoclonal antibodies – Bovine

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Introduction

The subcommissural organ (SCO) is a midline ependymal gland located in the roof region of the third ventricle contiguous with the aqueduct of Sylvius (Leonhardt 1980; Rodríguez et al. 1992). The SCO secretes *N*-linked complex-type glycoproteins (Herrera and Rodríguez 1990) of high molecular weight (Nualart et al. 1991; Grondona et al. 1994; López-Avalos et al. 1996). The bulk of this secretion is released into the ventricular cerebrospinal fluid (CSF), where it condenses to form an evergrowing thread-like structure known as Reissner's fiber (RF; Oksche 1969, 1993; Sterba 1969). RF grows caudally by addition of newly released glycoprotein molecules to its rostral end, and extends along the aqueduct, fourth ventricle, and the whole length of the central canal of the spinal cord. At the end of the central canal, in the filum, the constituent glycoproteins of RF become unpacked, undergo chemical changes, and appear to reach local blood capillaries (Olsson 1955; Hofer et al. 1984; Rodríguez et al. 1987). Thus, RF can be regarded as a polymerized pool of secretory material released by the SCO into the CSF. Indeed, polyclonal (Sterba et al. 1982; Rodríguez et al. 1984) and monoclonal (Pérez et al. 1995) antibodies raised against the glycoproteins extracted from the bovine RF selectively immunostain the secretory cells of the SCO.

On the other hand, polyclonal antibodies raised against secretory glycoproteins extracted from the SCO proper immunostain the secretory cells of the SCO and the RF (Rodríguez et al. 1985; Grondona et al. 1994). Polyclonal antibodies against secretory glycoproteins extracted from the bovine RF and the bovine SCO have revealed the presence of immunoreactive ependymal cells in the central canal of the bovine spinal cord (Rodríguez et al. 1985). These cells increase in number in a ventro-caudal direction, being most numerous in the lumbo-sacral region. In this latter region, immunoreactive material is also found in globular structures located in the lumen of the central canal, and on the luminal surface of the ependymal lining. Rodríguez et al. (1985)

have considered the possibility that the immunoreactive ependymal cells either absorb or secrete an RF constituent. Based on a scanning-electron-microscopic study, Tulsi (1982) has suggested that cell elements of the sacral central canal secrete a material that becomes part of RF. Because of the polyclonal nature of the antibodies raised against the bovine RF (Rodríguez et al. 1984), it is possible that the immunoreaction seen in the ependymal cells of the bovine central canal is the result of antibodies against contaminats in the RF preparation. This is indeed suggested by the immunoreaction of the luminal globular structures that correspond to protrusions of ependymal cells (Rodríguez et al. 1985).

We have recently obtained a set of ten monoclonal antibodies using bovine RF-glycoproteins as immunogens. These monoclonal antibodies have been characterized immunocytochemically (Pérez et al. 1995). In the present investigation, we report the characterization of three of these monoclonal antibodies by enzyme immunoassay and immunoblotting. These three antibodies have been used to re-investigate the central canal of the bovine spinal cord with the aim of establishing whether the immunoreactive material present in the ependymal cells is related to the RF-glycoproteins.

Materials and methods

Production of monoclonal antibodies

Preparation of antigen. For the present investigations, 4 mg RF-glycoproteins were obtained from approximately 400 cows. RF was collected by perfusing the central canal of the bovine spinal cord with artificial CSF (Rodríguez et al. 1984) and was extracted in a medium containing 50 mM ammonium bicarbonate, pH 8.0, 1 mM EDTA, and 0.5 mM phenyl-methyl sulfonyl fluoride. Aliquots of 10 µg glycoproteins were stored at –20° C.

Immunization. The following protocol was used for the immunization of female mice (Balb/c; Charles River). RF-glycoproteins (20 µg) were dissolved in 125 µl 0.1 M phosphate-buffered saline (PBS) and mixed with 125 µl Freund's complete adjuvant. At day 0, this solution was injected subcutaneously at four different sites of four mice (12 weeks old). At day 30, 125 µl PBS containing 20 µg glycoproteins were emulsified in 125 µl of Freund's incomplete adjuvant and injected at four subcutaneous sites. At day 45, a similar amount of proteins emulsified in incomplete Freund's adjuvant was injected intraperitoneally. At day 55, blood samples were obtained from a tail vein. The serum (pre-fusion) was screened by immunocytochemistry of the bovine SCO and RF, and by enzyme-linked immunosorbent assay (ELISA; see below). The best responder mouse was selected to continue the immunization and fusion. The final booster was at day 67. It consisted of a morning intraperitoneal injection and an afternoon intravenous injection (tail vein) of 20 µg glycoproteins in 125 µl of PBS. Fusion was at day 70.

Fusion. Spleen cells of the immunized mouse were fused with cultured myeloma cells (P3-X63 Ag 8653) at a ratio of 1:1, by the gradual addition of polyethylene glycol 4000 (50% w/v, Merck; Galfre et al. 1977). The hybridoma cells thus obtained were diluted with HAT-20 medium (hypoxanthine-aminopterin-thymidine, plus 20% fetal calf serum) to obtain a concentration of 2×10^5 cells/ml. They were distributed into five 96-well microtiter plates which had previously (1 day before) been incubated with 10^3 mouse peritoneal macrophages (feeder cells) per well cultured in HAT medium. One line of eight wells was used to culture only myeloma cells used as a test for the HAT medium; a second line of eight wells was used to culture only spleen lymphocytes in HAT medium. The supernatant of these wells was used as a control when screening the supernatants produced by the hybridoma cells. Cells were cultured at 37° C, in an atmosphere of 5% CO_2.

Screening of supernatant media. This screening was performed by using supernatant medium (50 µl) collected from each well, starting from the 10th day of culture, and an indirect ELISA method. For this purpose, 96-well flat-bottomed microtiter plates (styrene, high binding, Costar, Cambridge, USA) were used. Each well was coated with 50 µl of a solutions consisting of 5 µg/ml RF-glycoproteins in 0.1 M PBS, pH 7.4, overnight at 4° C. Subsequently, each well was incubated with: (1) 300 µl blocking solution, viz., PBS containing 0.25% bovine serum albumin (BSA), and 0.05% Tween 20, for 2 h at room temperature; (2) 50 µl hybridoma supernatant medium, for 2 h, negative control wells (3/plate) being incubated with the supernatant from the spleen lymphocyte wells, whereas positive control wells were incubated with the pre-fusion serum (1:1000 and 1:10000 dilution); (3) 50 µl anti-mouse IgG conjugated with horseradish peroxidase (HRP; Sigma, St. Louis, USA) at a 1:1000 dilution, for 1 h; (4) 50 µl 0.1 M acetate buffer, pH 6, containing 0.01% tetramethyl benzidine (Sigma) and 0.06% perhydrol (Merck, Darmstadt, Germany) for 5 min. The chromogen reaction was stopped by addition of 50 µl 2 M sulfuric acid. After each incubation (steps 1–4), the wells were washed with 0.9% NaCl containing 0.05% Tween 20. The optical density was determined using a microplate reader (Microplate Reader 2001, BioWhittaker, Walkersville, USA), at 450 nm. This ELISA procedure did not identify monoclonal antibodies corresponding to IgA or IgM. Those hybridoma colonies producing specific antibodies were immediately expanded by transferring them into 24-well plates containing in each well 1 ml HT medium and 50000 feeder cells. One or two days later, the supernatants were screened by ELISA. Those colonies still producing specific antibodies were used for cloning.

Cloning and expansion. Each of the colonies from the 24-well plate producing specific antibodies was progressively diluted with HT medium and then cultured in 96-well plates at an averaged density of 1 hybridoma cell per well, containing peritoneal macrophages as feeder cells. After 12 days, the supernatants were screened by ELISA. The cells from positive wells were further expanded (24-well plate) and cloned (96-well plate) once more. After this second expansion-cloning step, all wells containing growing hybridoma cells had specific antibodies, thus indicating that monoclonal antibodies had been obtained.

Three monoclonal antibodies were obtained: Mabs 2A5, 3B1, and 3E6. Large amounts of each of them were obtained by culture of the selected cell lines in RPMI-10 medium, in large culture flasks. The immunoglobulins were characterized by double immunodiffusion, by using antibodies against the subtypes of IgG (Sigma). Mabs 2A5 and 3B1 were IgG1; Mab 3E6 was IgG2a.

Purification and labelling of the monoclonal antibodies. Monoclonal antibodies were purified from the culture medium by affinity chromatography using protein A-sepharose for the IgG2a subtype and protein G-sepharose (Pharmacia LKB, Uppsala, Sweden) for the IgG1 subtypes. Purified monoclonal antibodies were labelled with HRP type VI (Sigma) by using the periodate method of Wilson and Nakane (1978).

Characterization of monoclonal antibodies. The three monoclonal antibodies were characterized by three different ELISA protocols, immunocytochemistry, and immunoblotting of RF-glycoproteins.

Direct enzyme immunoassay (affinity ranking)

The wells of a microtiter plate were coated with 50 µl 5 µg/ml RF-glycoproteins in 0.1 M PBS, pH 7.4. Each well was then se-

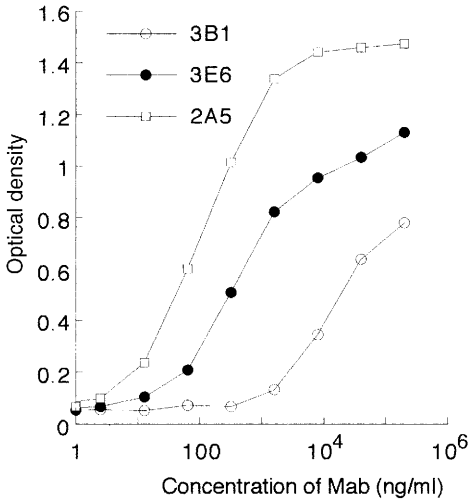


Fig. 1. Direct ELISA method. The wells were coated with Reissner's fiber (RF) glycoproteins; Mabs 3B1, 3E6 and 2A5 were then used at increasing concentrations. An affinity ranking is clearly seen, with Mab 2A5 displaying the highest affinity

quentially incubated with: (1) 300 µl blocking solution (see above); (2) monoclonal antibody purified by affinity chromatography and labelled with peroxidase; and then processed (3) for the diaminobenzidine reaction (see above). A series of increasing concentration (1–10^6 ng/ml) was used for each monoclonal antibody. Each concentration was tested in duplicate. Wells coated with BSA were used as controls. To establish an affinity ranking among the three monoclonal antibodies tested, the concentration giving 50% of maximal binding was determined for each (Fig. 1). According to this criterion, Mab 2A5 had the highest affinity and Mab 3B1 the lowest (Fig. 1).

Competitive enzyme immunoassay (competition test)

For this purpose, wells were coated with RF-glycoproteins at a concentration of 4 µg/ml and then incubated with PBS containing BSA and Tween 20. This was followed by incubation with unlabelled Mab 3E6 at concentrations ranging from 0–10^4 ng/ml (Fig. 2). After being washed, the wells were exposed either to HRP-labelled Mab 3E6 (control antibody) or to labelled test Mabs 2A5 or 3B1 (Fig. 2). Control and test monoclonal antibodies were used at a concentration of 1 µg/ml. The same competition test was performed for Mab 2A5 (Fig. 3).

Antibody sandwich enzyme immunoassay

One of the monoclonal antibodies was used as the capture antibody. RF-glycoproteins were used as the antigen. HRP-labelled monoclonal antibody (1 µg/ml), which was different from the capture antibody, was used as bound antibody (Fig. 4). Two negative controls were used: wells coated with non-specific mouse IgG (Sigma) instead of the capture monoclonal antibody, and incubation with a non-related antigen (BSA).

Immunocytochemistry

The three monoclonal antibodies were characterized by light- and electron-microscopic immunocytochemistry of the SCO of the bovine, pig, and rat. This study has been reported separately (Pérez et al. 1995).

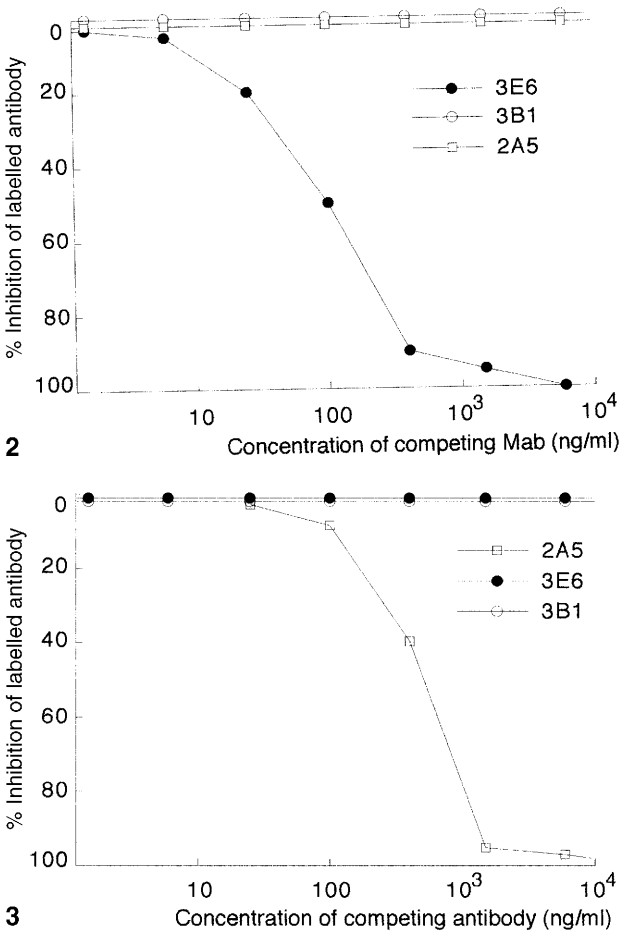


Fig. 2. Competitive ELISA. The wells were coated with RF-glycoproteins and incubated first with increasing concentrations of unlabelled Mab 3E6, and then with a fixed concentration (1 µg/ml) of labelled Mabs 3E6, 3B1, or 2A5. Mab 3E6 does not compete with the other two Mabs for binding to RF-glycoproteins

Fig. 3. Competitive ELISA. Same procedure as in Fig. 2, but the unlabelled antibody was Mab 2A5. This antibody does not compete with Mabs 3E6 and 3B1 for the binding to RF-glycoproteins

Immunoblotting

Bovine RF extracted in ammonium bicarbonate (Nualart et al. 1991) was processed for SDS-polyacrylamide gel electrophoresis according to Laemmli (1970). A 5–15% polyacrylamide linear gradient was used. Samples of 2.5-µg RF-proteins were loaded onto the stacking gel. The gels were electrotransferred onto Immobilon paper (Millipore, Bedford, USA) according to the method of Towbin et al. (1979). The blots were saturated with 5% non-fat milk in 0.1 M PBS containing 0.15 M NaCl and processed for immunostaining using the following primary antibodies: (1) a polyclonal antibody raised in rats against the bovine RF-glycoproteins extracted in ammonium bicarbonate (Nualart and Rodríguez 1996) and diluted 1:1000; (2) Mabs 3B1, 2A5, and 3E6, at a dilution of 10 µg/ml. Incubation was at room temperature, overnight. The blots were subsequently incubated with anti-rat IgG (when anti-RF was used) or anti-mouse IgG, and rat PAP or monoclonal mouse PAP. 4-Chloro-1 naphthol was used as electron donor for the peroxidase reaction. Controls included incubation of blots with pre-immune rat serum or with purified mouse IgG (Sigma), or omission of the primary antibody. Immunoblotting was carried out four times, using two RF preparations.

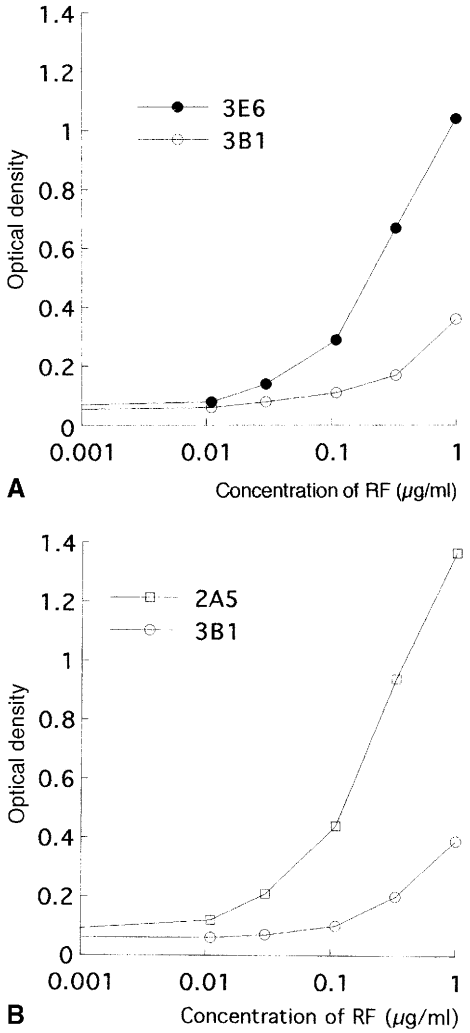


Fig. 4A, B. Antibody sandwich ELISA. **A** Wells were coated with Mab 2A5 (capture antibody) and incubated first with increasing concentrations of RF-glycoproteins, and then with a fixed concentration of labelled Mabs 3E6 or 3B1 (bound antibodies). **B** Mab 3E6 was the capture antibody and Mabs 2A5 and 3B1 the bound antibodies

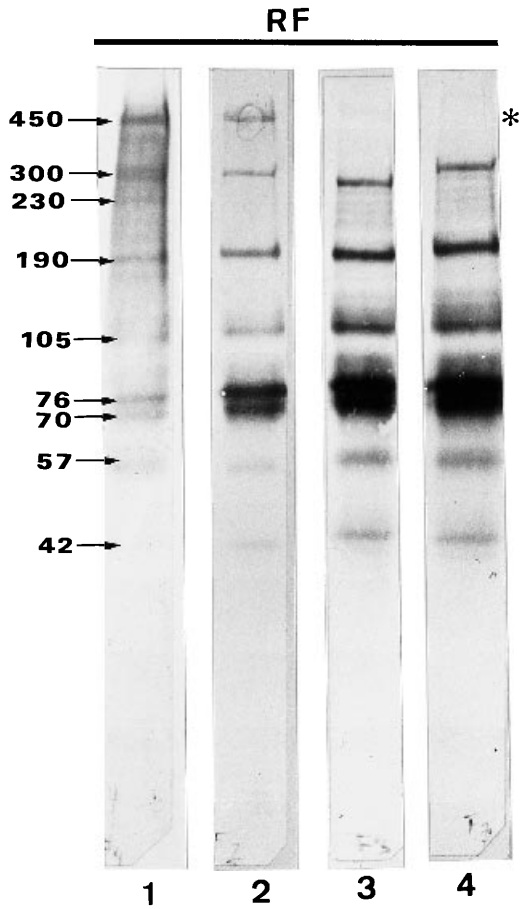


Fig. 5. Immunoblotting of an extract of bovine Reissner's fiber (*RF*) using a polyclonal antiserum against bovine RF (*lane 1*) and Mabs 3E6 (*lane 2*), 3B1 (*lane 3*), and 2A5 (*lane 4*). *Left*, molecular weights in kDa; *asterisk*, absence of the 450-kDa band in *lanes 3* and *4*

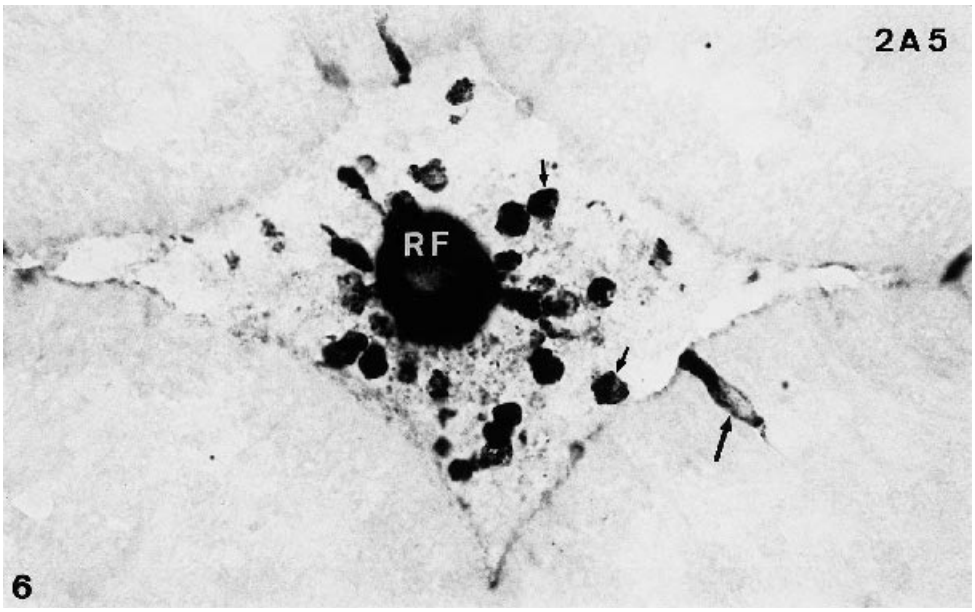
Immunocytochemical study of the bovine RF and central canal of the spinal cord

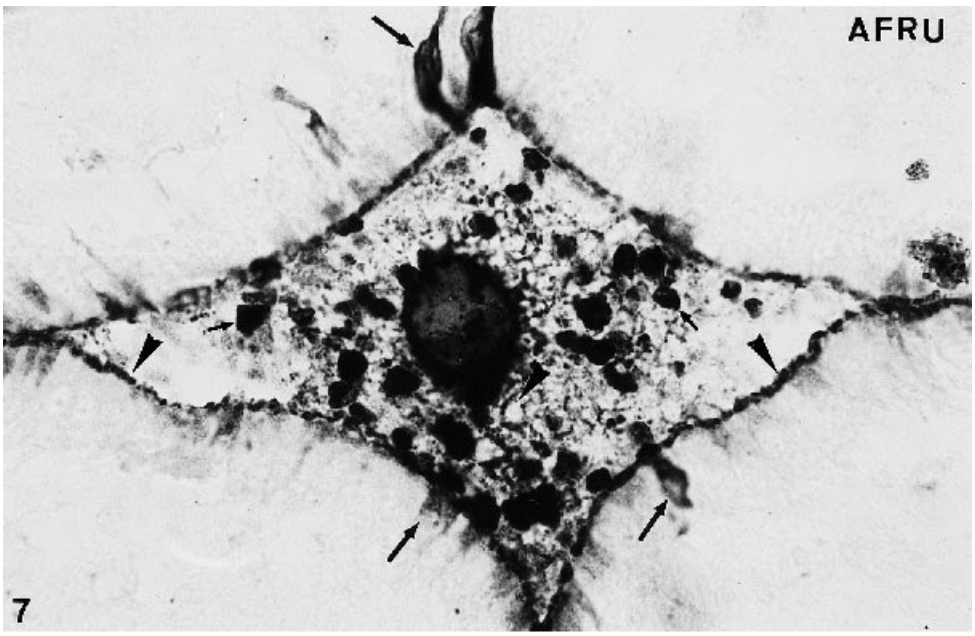
The lumbosacral region of the bovine spinal cord was fixed by immersion in Bouin's fluid for 2 days. A series of adjacent paraffin sections, 8 μm thick, were mounted on polylysinated slices. Adjacent sections were processed for the immunoperoxidase method of Sternberger et al. (1970) using the following primary antibodies: (1) a polyclonal antibody, raised in rabbits, against the bovine RF-glycoproteins extracted in a medium containing dithiothreitole, EDTA, and urea (AFRU; Rodríguez et al. 1984) and used at a 1:1000 dilution; (2) Mab 2A5; (3) Mab 3E6; (4) Mab 3B1. The monoclonal antibodies were used at 50 μg/ml. Anti-rabbit IgG (1:30 dilution) or anti-mouse IgG (1:50 dilution) were used as the secondary antibody. Sections were incubated for 45 min in rabbit PAP (1:75 prepared in our laboratory in Valdivia) or in a monoclonal mouse PAP (1:200 dilution, Sigma). This was followed by the diaminobenzidine reaction. Some of the sections were processed for sequential immunostaining. The sections were incubated with one of the monoclonal antibodies, anti-mouse IgG, and mouse PAP. After the diaminobenzidine reaction, the sections were mounted and photographed, and then treated with Gomori's oxidizing solution (González and Rodríguez 1980); this procedure removed the peroxidase reaction product and eluted the antibodies. The same sections were then incubated with AFRU, anti-rabbit IgG, and rabbit PAP, and re-photographed. In some sections, the sequence of incubation was AFRU first, and then the monoclonal antibody.

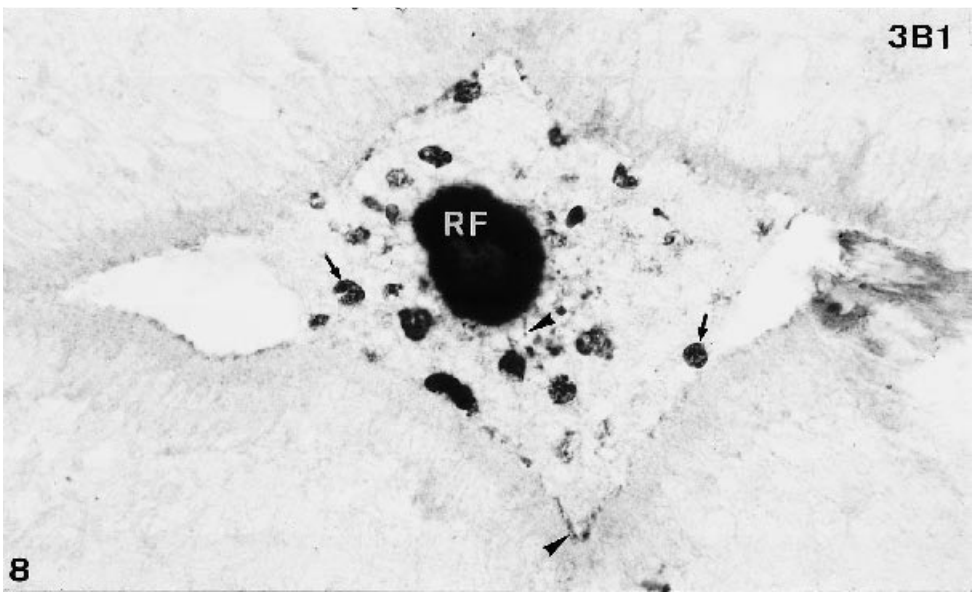
Results

Characterization of monoclonal antibodies

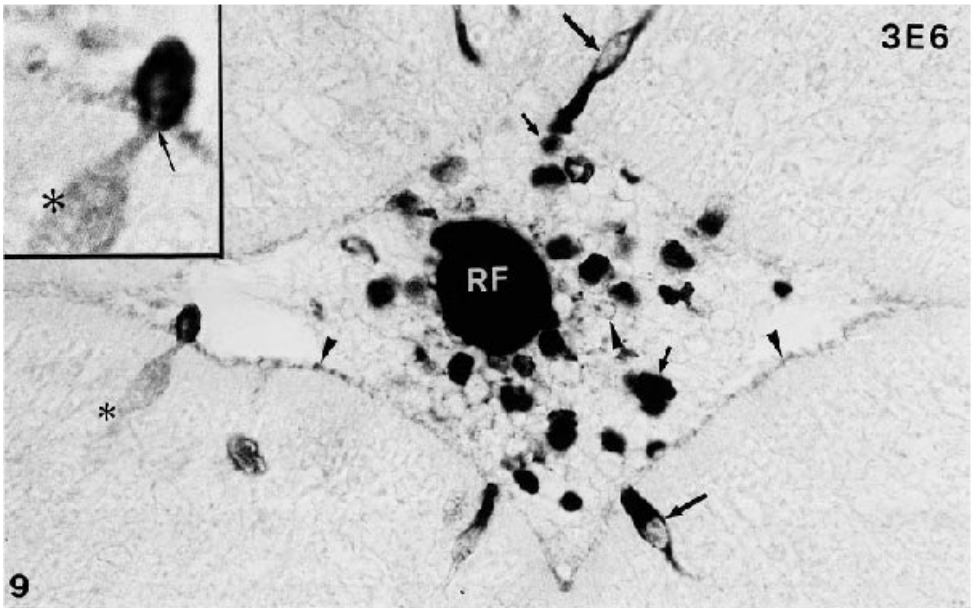
Competitive ELISA. When serial concentrations of unlabelled Mab 3E6 were allowed to bind to RF-glycoproteins, the HRP-labelled Mabs 3B1 and 2A5 displayed maximal binding throughout the whole range of the unlabelled Mab 3E6 concentrations; however, binding of labelled Mab 3E6 decreased progressively as the concentration of unlabelled Mab 3E6 increased (Fig. 2). Thus, Mab 3E6 did not compete for binding to RF-glycoproteins with Mabs 3B1 and 2A5. The same procedure showed that Mab 2A5 did not compete with Mabs 3E6

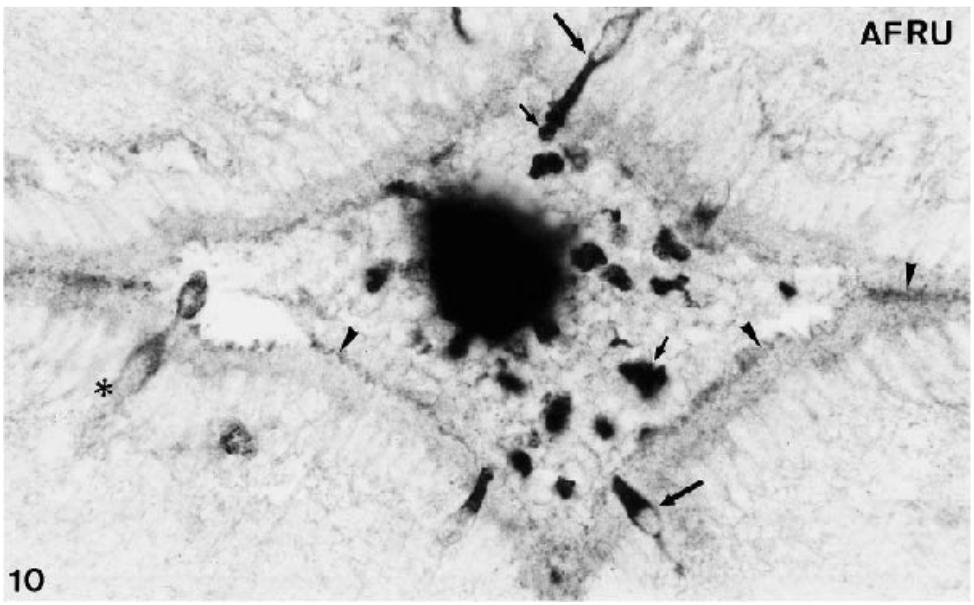






Figs. 6–8. Three adjacent serial sections of the bovine spinal cord, at the lower lumbar level, immunostained with Mab *2A5* (**Fig. 6)**, an antiserum (*AFRU*) against RF-glycoproteins (**Fig. 7**), and Mab *3B1* (**Fig. 8**). *RF*, Reissner's fiber; *large arrows*, immunoreactive ependymal cells; *small arrows*, immunostained globular structures lying in the lumen of the central canal; *arrowheads*, immunoreactive coat on the surface of the central canal and on the surface of unstained luminal globular structures. ×530





Figs. 9, 10. Transverse section of the spinal cord adjacent to that shown in Fig. 8. This section was processed for sequential immunostaining using Mab *3E6* in the first sequence (**Fig. 9**), and an antiserum against RF-glycoproteins (*AFRU*) in the second sequence (**Fig. 10**). *RF*, Reissner's fiber; *large arrows*, immunoreactive ependymal cells; *small arrows*, immunostained globular structures lying in the lumen of the central canal; *arrowheads*, immunoreactive coat on the surface of the central canal and on the surface of unstained luminal globular structures; *asterisk*, immunoreactive ependymal cell shown at higher magnification in *insert*. ×530. *Insert:* Detailed magnification of one of the immunoreactive ependymal cells shown in Fig. 9. *Asterisk*, Cell body; *arrow*, terminal protruding into the central canal. ×1300

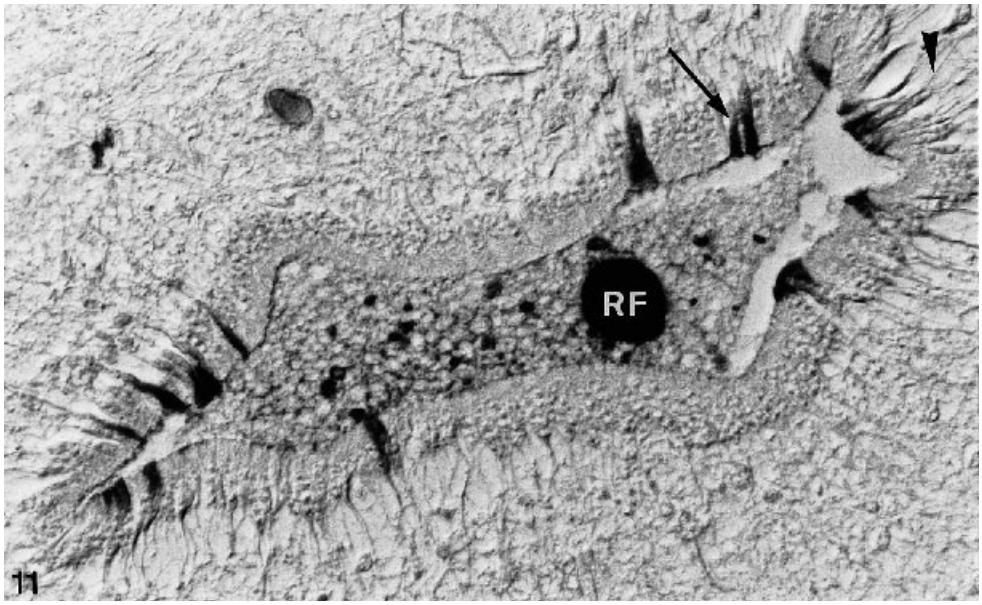
and 3B1 (Fig. 3). These results indicated that Mabs 3E6, 2A5, and 3B1 were directed against different epitopes, and that the latter were not close enough to interfere with the binding of the corresponding monoclonal antibody.

Antibody sandwich ELISA. By using Mab 2A5 as capture antibody and Mab 3E6 and 3B1 as bound antibody, the signal produced by Mab 3E6 was several fold higher than that produced by Mab 3B1 (Fig. 4A). When using Mab 3E6 as capture antibody and Mab 2A5 and 3B1 as bound antibody, Mab 2A5 produced a signal several fold higher than that produced by Mab 3B1 (Fig. 4B).

Immunoblotting. The polyclonal anti-RF serum revealed a banding pattern similar to that shown by other anti-bovine RF sera (cf. Nualart et al. 1991; Nualart and Rodríguez 1996). Bands of 450, 300, 230, 190, 105, 76, 70, and 57 kDa were revealed by this antiserum, the 450-kDa band being the most strongly labelled (Fig. 5). Mab 3E6 demonstrated the same banding pattern as the polyclonal antibody, the only difference being that the 76-kDa and 70-kDa bands were strongly labelled by this monoclonal antibody (Fig. 5).

Mabs 3B1 and 2A5 produced similar banding patterns (Fig. 5). However, these patterns differed from those revealed by Mab 3E6 and the polyclonal antibody. A consistent difference detected in the four immunoblots performed was that Mabs 3B1 and 2A5 did not label the 450-kDa band (Fig. 5). Furthermore, the bands of 57 and 42 kDa, which were readily and consistently revealed by Mabs 3B1 and 2A5, were weakly reactive with Mab 3E6 (Fig. 5).

Immunocytochemistry of the bovine RF and central canal of the spinal cord The three monoclonal antibodies and AFRU strongly stained the RF (Figs. 6–9). The



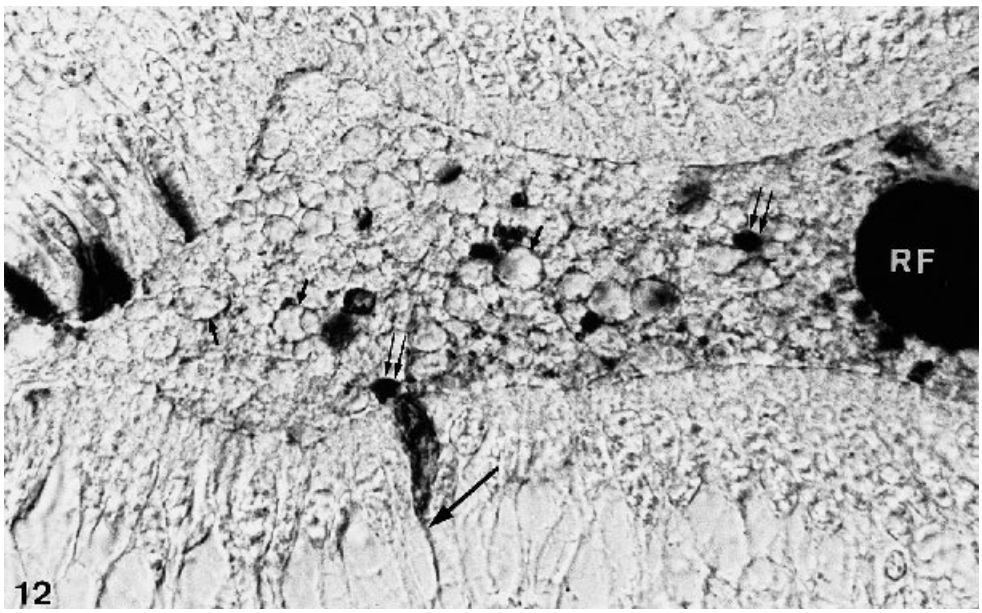


Fig. 11. Transverse section of the bovine spinal cord at the sacral level, immunostained with Mab 2A5 and photographed using interference phase contrast. *RF*, Reissner's fiber; *arrow*, immunoreactive ependymal cell; *arrowhead*, basal processes of stained ependymal cells. ×430

Fig. 12. Higher magnification of Fig. 11. *RF*, Reissner's fiber; *large arrow*, basal process of an immunoreactive ependymal cell; *double arrows*, ventricular protrusions of immunoreactive ependymal cells; *small arrows*, coat of immunoreactive material on surface of unstained luminal spherical structures. ×860

staining of adjacent sections with AFRU and the three monoclonal antibodies showed that the number and distribution of the cells reacting with AFRU and Mabs 2A5 and 3E6 were similar (Figs. 6, 7, 9). Sequential staining of the same section demonstrated that the same ependymal cells were stained by AFRU, Mab 2A5, and Mab 3E6 (Figs, 9, 10). None of the ependymal cells reacted with Mab 3B1 (Fig. 8).

The immunoreactive ependymal cells were bipolar, with a thin basal process (Figs. 11, 12), and a thick apical process displaying a neck region and a dilated globular ending protruding into the lumen of the central canal (Figs. 7, 9, 12). The terminals protruding into the central canal were strongly reactive with AFRU (Fig. 10), Mab 3E6 (Fig. 9), and Mab 2A5 (Fig. 6); these terminals, but not the cell body, were also reactive with Mab 3B1 (Fig. 8).

At lower lumbar and sacral levels, the lumen of the central canal was mostly occupied by globular structures that appeared to correspond to detached protrusions of the ependymal cells (cf. Rodríguez et al. 1985). A small proportion of these globular structures reacted with AFRU and the three monoclonal antibodies (Figs. 11, 12), thus suggesting that they corresponded to protrusions of the immunoreactive ependymal cells.

The free surface of the non-reactive ependymal cells and the free surface of the non-reactive globular structures (probably corresponding to protrusions of the non-reactive ependymal cells) were covered by a thin layer of material strongly immunoreactive with AFRU (Fig. 7; cf. Rodríguez et al. 1985). This coat reacted weakly with the three monoclonal antibodies (Figs. 6, 8, 9, 12).

Discussion

Monoclonal antibodies against the secretory products of the SCO have previously been obtained by using crude extracts of the SCO of bovine (Meiniel et al. 1988) or chick embryos (Didier et al. 1992) as the immunogen; these extracts contain the precursor and processed forms of the secretory compounds. The present investigation reports the production of monoclonal antibodies against the constituent glycoproteins of the bovine RF, that is the processed forms of the material secreted by the SCO.

We have produced ten monoclonal antobodies that we have characterized immunocytochemically and used for a light- and electron-microscopic immunocytochemical study of the bovine SCO (Pérez et al. 1995). We report here the procedure for the production of these monoclonal antibodies and the characterization of three of them by three types of solid-phase assays (ELISA) and by immunoblotting of bovine RF-extracts. The competitive and the sandwich ELISA methods demonstrate that Mabs 3E6, 2A5, and 3B1 are directed against different epitopes, and that such epitopes are not close enough to interfere with the binding of the corresponding monoclonal antibody. Furthermore, each of the three monoclonal antibodies has a distinct affinity under ELISA conditions. The observation that these antibodies immunoreact in tissue sections with the secretory material present in the bovine SCO (Pérez et al. 1995) and RF indicates that the three epitopes are present in RF constituents secreted by the SCO. This is further supported by the evidence that these antibodies strongly label RF-glycoproteins under blotting conditions. The present immunoblotting findings do not indicate whether a given RF constituent lacks one or more of the epitopes detected by these antibodies. Thus, although the 450-kDa glycoprotein is labelled by Mab 3E6 and not by the two other monoclonal antibodies, this could be the result of the lack of accessibility of the latter to the corresponding epitope, under blotting conditions. The difficulties of achieving immunoreaction of monoclonal antibodies under blotting conditions are well known (Ey and Ashman 1986; Lane et al. 1989). Nevertheless, the interesting possibility that the 450-kDa compound lacks the epitopes reacting with Mabs 3B1 and 2A5 must be considered.

Mab 3E6 and the polyclonal anti-RF serum AFRU share several characteristics (Table 1). Mabs 2A5 and 3B1 share the same immunoblotting properties, but display different immunocytochemical properties in the SCO (Pérez et al. 1995) and in the ependymal cells of the central canal (see below). The results obtained with the monoclonal antibodies when using three methods of analysis, namely, ELISA, immunocytochemistry, and blotting, reveal a high degree of discrepancy. However, the characterization of these antibodies by these three methods has allowed us to perform the present immunocytochemical study of the central canal.

The polyclonal AFRU, when used for immunostaining of paraffin sections of the bovine spinal cord reacts with (1) the RF; (2) a population of ependymal cells; (3) spherical structures occupying most of the canal lumen; and (4) a thin layer on the luminal surface of the ependymal cells (Rodríguez et al. 1985; present report). The immunoreactive spherical structures and ependymal cells are more numerous in the lumbosacral regions (Rodríguez et al. 1985). Taking into account that, for the preparation of the anti-RF serum, RF is collected by perfusing the central canal with artificial CSF, Rodríguez et al. (1985) have considered the possibility that such a serum might contain antibodies against substances that are present in structures of the central canal but that are different from RF. These additional antibodies might thus be responsible for the immunostaining of structures other than RF by AFRU. This possibility can, however, be ruled out by the following two findings: (1) an antiserum raised against secretory glycoproteins extracted from the bovine SCO proper immunostains the RF, luminal spherical structures, and a population of ependymal cells, similar in number and distribution to that shown by AFRU (Rodríguez et al. 1985); (2) Mabs 2A5 and 3E6, raised against an RF extract, immunostain the secretory material of the SCO (Pérez et al. 1995), the RF, luminal spherical structures, and the same population of ependymal cells revealed by AFRU (present findings). Therefore, identical or similar epitopes reacting with these two monoclonal antibodies are present in the SCO, RF, and some ependymal cells of the central canal.

On the other hand, there are differences between the SCO-RF material and the AFRU-immunoreactive material of the ependymal cells. Thus, Mab 3B1, which reacts with the secretion of the SCO (Pérez et al. 1995) and with RF, does not react with the ependymal cells. Furthermore, the material in the immunoreactive ependymal cells, unlike the SCO-RF material, is not stainable with the paraldehyde-fuchsin method of Gomori or the periodic-acid Schiff method (Rodríguez et al. 1985). The existence of homologies (reactivities to AFRUand two monoclonal antibodies) and differences between the

Table 1. Immunoreactive properties of a polyclonal antibody and three monoclonal antibodies raised against Reissner's fiber glycoproteins

	2A5	3B1	3E6	AFRU
Ranking affinity	+++	+	++	+++[a]
Immunoblot of RF (band range in kDa)	300–42	300–42	450–42	450–70
IMC SCO[b]	RER-sg	sg	sg	RER-sg
IMC RF	+++	+++	+++	+++
Ependymal cells	+++	–	+++	+++
Epend. protrusions	+++	+++	+++	+++
Spherical structures in central canal	+++	+++	+++	+++
Ependymal coat	+	+	++	+++

2A5, 3B1, 3E6, Monoclonal antibodies; AFRU, polyclonal antiserum against bovine Reissner's fiber glycoproteins (Rodríguez et al. 1985); SCO, subcommissural organ; RF, Reissner's fiber; IMC, immunocytochemistry; RER, rough endoplasmic reticulum; Sg, secretory granules; +, ++, +++, degree of immunoreactivity; –, lack of immunoreactivity

[a] Result not shown

[b] from Pérez et al. 1995

SCO-RF material and the material present in some ependymal cells leads to the conclusion that both materials share a partial chemical identity.

Mabs 2A5 and 3B1 reveal the same banding pattern in immunoblot but behave in a different way with respect to the immunostaining of the ependymal cells of the central canal, with Mab 2A5 producing the labelling of these cells and Mab 3B1 being non-reactive. This again favors the view of a partial chemical identity between the RF-glycoproteins and the material present in the ependymal cells. An alternative explanation would be that only a fragment of the RF-glycoproteins, bearing the 2A5 and 3E6 epitope but lacking the 3B1 epitope, is present in the ependymal cells.

The important question whether the immunoreactive ependymal cells secrete or absorb the immunoreactive material has not been solved by the present investigation (for a detailed discussion of this aspect, see Rodríguez et al. 1985). Some findings indicate the secretory nature of these cells. Cells displaying ultrastructural characteristics of elements involved in the secretion of peptide(s) and/or proteins are present in identical regions of the central canal of the bovine spinal cord (Rodríguez et al. 1985). Based on scanning-electron-microscopic studies, Tulsi (1982) has suggested that products secreted by ependymal cells in the sacral spinal cord are added to RF in the possum. Results obtained in the infundibular organ of the amphioxus *Branchiostoma lanceolatum* by means of antisera against bovine RF and bovine SCO extracts have led Olsson et al. (1994) to suggest that the bovine RF is formed by products secreted by two different sources, viz., the SCO and a second, not yet identified, secretory structure. They consider that the AFRU-immunoreactive ependymal cells are the second possible source. The immunocytochemical behavior of the AFRU-immunoreactive ependymal cells with Mab 3B1, viz., labelling of the luminal protrusion and the lack of labelling of the other cytoplasmic regions, is an indication that the material in the protrusion is a processed secretory form. Interestingly, in the bovine SCO, Mab 3B1 only reacts with granules present in the ventricular cell pole (Pérez et al. 1995).

The strongly AFRU-immunoreactive coat on the surface of the central canal is weakly labelled by the monoclonal antibodies, suggesting that the origin of this material is not the ependymal cells; it could correspond to material released from RF, either as a postmortem artefact (cf. Rodríguez et al. 1985) or as a physiological phenomenon. The absence of such a coat in specific regions, such as the cervical spinal cord and filum terminale (Rodríguez et al. 1985), supports the latter possibility.

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