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Single injection into the cerebrospinal fluid of antibodies against the secretory material of the subcommissural organ reversibly blocks formation of Reissner's fiber: immunocytochemical investigations in the rat

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Summary. An antibody (cf. Rodríguez et al. 1984b) raised in rabbits against the glycoproteins of the bovine Reissner's fiber (RF) was injected into the lateral brain ventricle of 38 rats with the aim to interfere with RF formation. The rats were killed 20 min; 1, 4, 8, 12 h; and 1, 2, 3, 5, and 8 days after the injection. Based on the fact that the material secreted by the subcommissural organ (SCO) into the cerebrospinal fluid (CSF) first condenses on the organ surface as a distinct layer (pre-RF material) and then becomes assembled to form RF and that both structures are distinguishable in tissue sections, three immunostaining procedures were applied. They served to visualize: (i) secretory material that had not bound the injected antibody; (ii) secretory material-antibody complexes formed in vivo; and (iii) antibody not bound to its antigen and present in the ventricles and the subarachnoid space. After a single injection of the above-mentioned antibody the following events were observed: (1) The antibody was present in the brain cavities for at least 8 h. (2) The injected antibody bound selectively to the pre-RF and RF. (3) Pre-RF displayed antibody binding during the 24 h following the injection. During the 2nd and 3rd post-injection days, the pre-RF was free of antibody, indicating that it was formed by newly released secretory material. (4) Approximately 4 h after the injection, the RF detached from the SCO and underwent fragmentation. Clusters of these fragments were found in the Sylvian aqueduct and fourth ventricle. (5) In the fragmented original RF the injected antibody against Reissner's fiber remained bound throughout the entire period of observation, i.e. for 8 days. (6) In rats of the 1-, 3-, 5- and 8-day-groups, RF was missing from the central canal of the spinal cord. (7) One day after the injection, a new RF structure started to grow from the rostral end of the SCO. This newly formed fiber could be distinguished from the original RF because of (i) its normal appearance; (ii) it did not display binding of the injected antibody. (8) At day 3, the growing RF had not yet extended to the Sylvian aqueduct. (9) At day 8, the new RF reached the fourth ventricle. Control experiments involved the intraventricular administration of (i) an antibody against the secretory material extracted from the entire bovine SCO; (ii) antivasopressin; and (iii) rabbit IgG. From these only antibody (i) bound to pre-RF and RF.

Key words: Subcommissural organ – Reissner's fiber – Secretory process – Experimental blockade – Immunocytochemistry – Rat (Sprague-Dawley, Holtzmann strain)

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Introduction

The subcommissural organ (SCO) is an ependymal structure of the brain highly specialized in secretion. It is located in the roof of the third ventricle at the entrance to the Sylvian aqueduct. The SCO secretes several glycoproteins (Rodríguez et al. 1987a). Some of these glycoproteins, and very probably all of them, are released into the third ventricle where they condense into a thread-like structure known as Reissner's fiber (RF) (cf. Sterba 1969). RF extends along the aqueduct, fourth ventricle and central canal of the spinal cord to end in a terminal dilation of the latter known as the "terminal ventricle" or "ampulla caudalis" (see Leonhardt 1980). By addition of newly released glycoproteins to its cephalic end RF continuosly grows in a rostral-caudal direction at a rather constant rate (cf. Ermisch 1973).

Although recent investigations have supported valuable information with respect to the nature and biosynthesis of the secretory products of the SCO (Rodríguez et al. 1986, 1987a; Meiniel et al. 1986, 1988), the function of the SCO–RF complex is still unknown. Several working hypotheses have been proposed with respect to the function of the SCO although none of them have been

Table 1. Experimental groups and immunostaining procedures used

Experimental group N°	Antibody injected into CSF	Immunostaining		
		Complete procedures; primary antibody used:		Incomplete procedure
		a	b	c
1	AFRU	AFRU	RAFRU	Anti-rabbit IgG→rabbit-PAP
2	ASO	AFRU	RAFRU	Anti-rabbit IgG→rabbit-PAP
3	Rabbit IgG	AFRU		Anti-rabbit IgG→rabbit-PAP
4	Anti-AVP	AFRU		Anti-rabbit IgG→rabbit-PAP
		↑	↑	↑
		Demonstration of SCO secretion + injected antibody	Demonstration of SCO secretion	Demonstration of antibodies injected in vivo

In each group, adjacent sections were stained with the complete procedure using AFRU (a) and RAFRU (b) as primary antibody, and with the incomplete procedure (c) in which incubation in a primary antibody was omitted

fully substantiated (see Leonhardt 1980; Severs et al. 1987). Considering that (i) the SCO is a structure formed by a single or a few layers of secretory cells, which line a rather irregular area of the ventricular wall, and (ii) the secretory cells are located at the borderline between the CSF and neural tissue and integrated with the latter in a very complex manner, it is not surprising to encounter serious difficulties when attempting to manipulate the SCO experimentally. Ablation of the SCO by electrolytic lesions necessarily implies damage to adjacent structures, especially to the posterior commissure and the pineal organ (Brown and Afifi 1965; Bugnon et al. 1965; Palkovits et al. 1965; Sallanon et al. 1984). Also, an opening of the ventricular cavity, followed by escape of CSF may lead to uncontrolled changes in CSF pressure and flow.

The availability of antibodies raised in rabbits against the secretory compounds of the SCO (Rodríguez et al. 1984b, 1988) provided us with a tool for experimentation that would selectively interfere with the functional activity of the SCO. For this purpose such antibodies were injected into the ventricular CSF of rats and their sites of binding were detected in tissue sections by use of the immunoperoxidase method employing an anti-rabbit IgG as the "primary antibody". We report here on the effect of a single injection of these antibodies on the formation of RF.

Materials and methods

Fifty-four male rats of the Holtzman (Sprague-Dawley) strain were used in the present experiments. All animals were anesthetized with ether. Subsequently, via a cannula stereotaxically positioned into the right lateral ventricle 20 µl of one of the antibody solutions (see below) was injected during a period of 20 min (rate 1 µl/min) by use of a perfusion pump. Coordinates for cannula placement within the lateral ventricle were: posterior from Bregma = 0.5 mm; lateral from sagittal suture = 1.5 mm; ventral from dura = 4.0 mm. In cases with the post-injection interval longer than 1 h, the rat was placed back into its cage and kept under constant temperature and photoregime (L:D = 12:12). These rats had free access to food and water. The following antibody solutions were used: (i) Antiserum against bovine Reissner's fiber developed in rabbits. For this antiserum the acronym AFRU (*A* = antiserum, *FR* = fiber of Reissner, *U* = urea) was introduced (see Rodríguez et al. 1984b). This antiserum was partially purified by precipitation with 2.0 M ammonium sulphate (pH 6.8, 22° C, 16 h) and centrifugation at 10 000 *g* (Clausen 1981). The precipitate was dissolved in phosphate-buffered saline (PBS), pH 7.4. The volume of PBS used to dissolve the precipitate was equivalent to half of the volume of the original serum. This resulted in a protein concentration of 10 µg/µl, as determined by the Lowry method. (ii) An antiserum against a partially purified extract of bovine SCO was developed in rabbit. For this antiserum the acronym ASO (A = antiserum, SO = subcommissural organ) is used (see Rodríguez et al. 1988). ASO was partially purified in the same way as indicated above for AFRU. (iii) Antiserum against arginine vasopressin (anti-AVP) developed in rabbits in our laboratory. (iv) Rabbit IgG, whole molecule, obtained from Sigma (St. Louis, MO., USA), dissolved in PBS at a protein concentration of approximately 8 µg/µl.

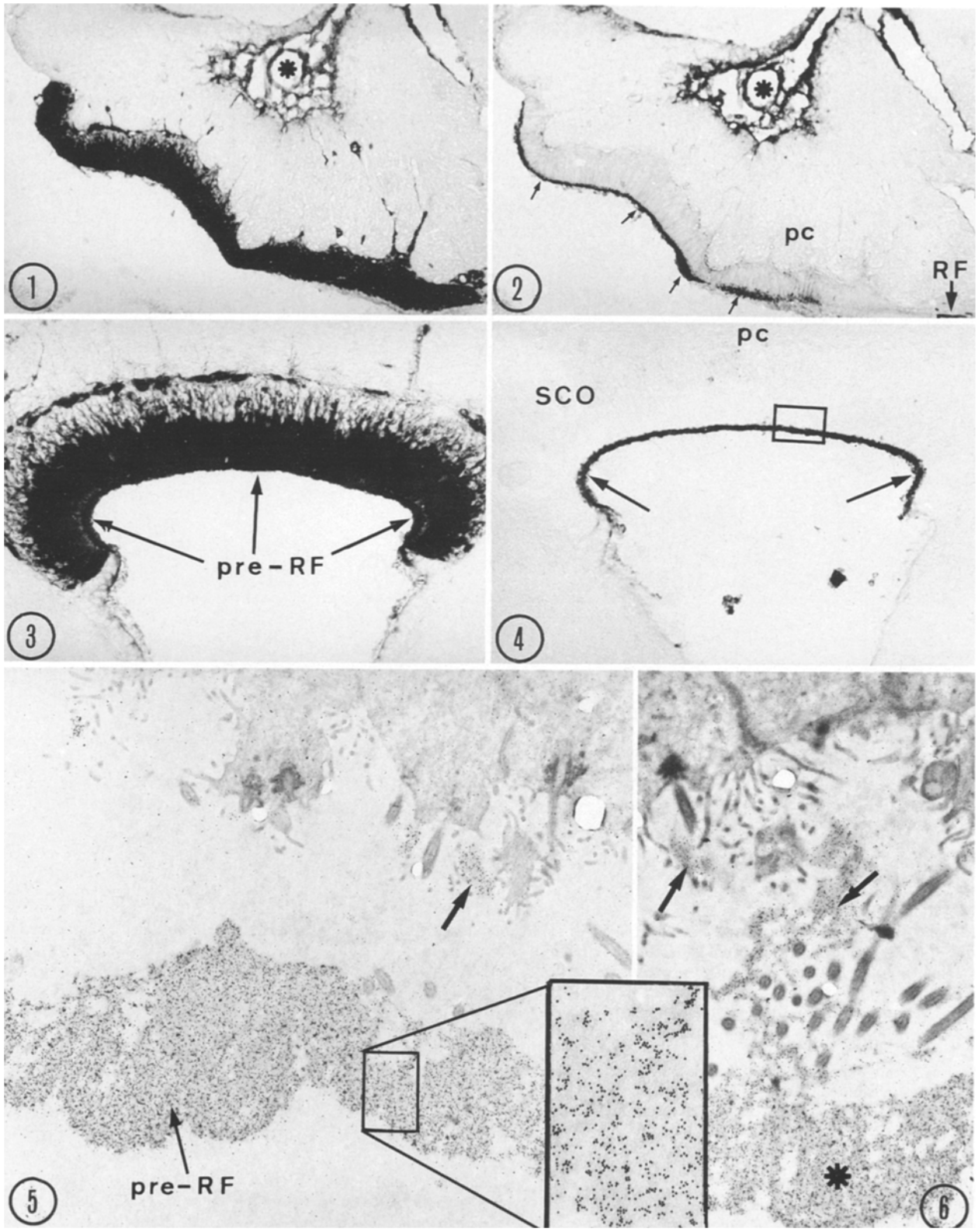
The 54 experimental rats were divided into the following groups: *Group 1:* 38 rats were injected with AFRU and killed at the following time intervals after the end of the 20 min perfusion: 0 min (3 rats), 20 min (3), 1 h (3), 4 h (2), 8 h (3), 12 h (2), 1 day (7), 2 days (2), 3 days (5), 5 days (4), 8 days (4). *Group 2:* 6 rats injected with ASO and killed at 0 min (2 rats), 20 min (2), 2 h (1) and 4 h (1) after perfusion. *Group 3:* 6 rats injected with rabbit IgG and killed 20 min (2), 2 h (2) and 8 h (2) after the injection. *Group 4:* 4 rats injected with anti-AVP and killed 20 min (2) and 2 h (2) after the injection.

Under ether anesthesia, the rats were first vascularly perfused with a washing solution followed by Bouin's fixative. The brains and the spinal cords were dissected out and immersed in Bouin's

Table 2. Demonstration of free antigen, Ag-Ab complexes and free antibody by the combined use of the three immunostaining protocols

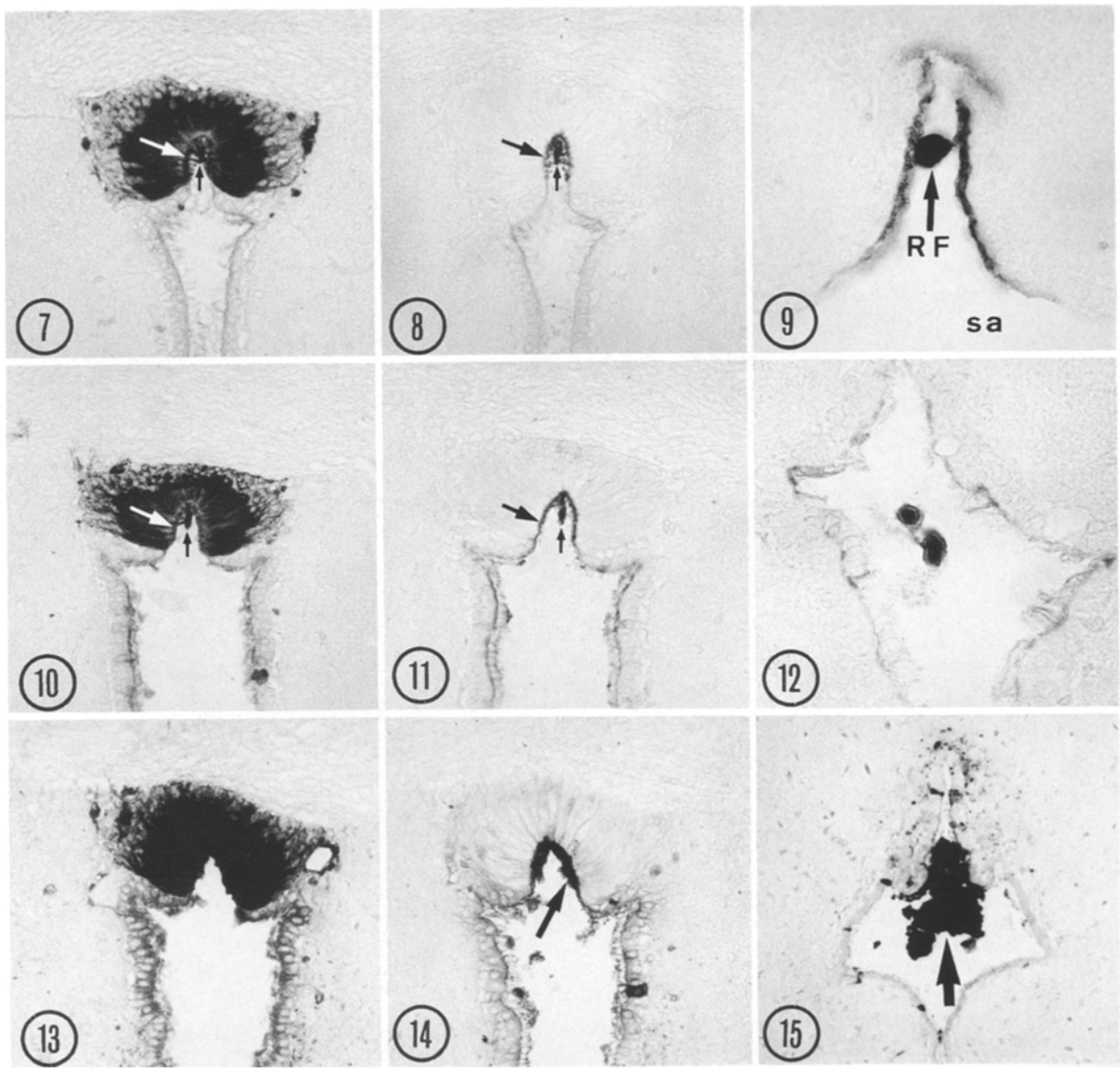
Complete method using AFRU	Incomplete method using anti-Rabbit IgG	Complete method using RAFRU
+	−	+ →Free antigen
+	+	+ →Ag-Ab complexes
+	+	− →Free Ab
+	ND	ND→Free Ag; Free Ab; Ag-Ab complexes
ND	+	ND→Ab either free or bound to Ag
ND	ND	+ →Ag either free or bound to Ab

+ = positive immunoreaction; − = negative immunoreaction, ND = immunostaining not done, Ag = antigen (SCO secretion), Ab = injected AFRU



Figs. 1–4. Rat 1 h after injection of AFRU into the CSF. **Figs. 1, 3.** Complete immunostaining procedure using AFRU as primary antibody. **Figs. 2, 4.** Incomplete immunostaining procedure using anti-rabbit IgG and rabbit-PAP. The injected AFRU appears bound to Reissner's fiber (RF), the pre-RF material (*arrows*) and also on structures of the subarachnoid space (*asterisk*). *SCO* subcommissural organ, *pc* posterior commissure, *rectangle* area similar to that shown in Fig. 5. **Figs. 1, 2.** Sagittal section through SCO. ×80. **Figs. 3, 4.** Cross section through the cephalic end of SCO. ×90.

Figs. 5, 6. Rat 20 min after injection of AFRU into the CSF. Incomplete immunostaining procedure using anti-rabbit IgG and protein A-gold of an area similar to that framed in rectangle of Fig. 4. Pre-RF (*asterisk*) and masses of extracellular secretory material (*arrows*) appear labeled. ×10000. *Insert*. Detailed magnification of pre-RF. The colloidal gold particles are shown. ×30000



Figs. 7–9. Rat at time zero after the intraventricular injection of AFRU. **Figs. 7, 8.** SCO immunostained with the complete (using AFRU as primary antibody) and incomplete procedures, respectively. *Thick arrow* pre-RF material, *thin arrow* RF. × 210. **Fig. 9.** RF (*arrow*) in the aqueduct (sa), immunostained with the incomplete procedure. × 500

Figs. 10–12. Rat 20 min after injection of AFRU into the CSF. **Figs. 10, 11.** SCO immunostained with the complete and incomplete procedures, respectively. *Thick arrow* pre-RF material, *small arrow* RF. × 210. **Fig. 12.** RF in Sylvian aqueduct stained with the incomplete method. × 420

Figs. 13–15. Rat 4 h after in vivo administration of AFRU into CSF. **Figs. 13, 14.** SCO stained following the complete and incomplete immunostaining protocols. *Arrow* pre-RF material. × 210. **Fig. 15.** Cluster of RF material in Sylvian aqueduct (arrow) immunostained with the complete method using AFRU as primary antibody. × 180

fluid for 2 days. After dehydration in increasing concentrations of ethanol the material was embedded in Paraplast. The area of the brain containing the SCO, aqueduct of Sylvius and fourth ventricle was serially sectioned. The cervical, thoracic and lumbar segments of the spinal cord were embedded separately. Each segment was divided into several pieces, which were embedded together in the same paraffin block and oriented accordingly to obtain cross sections.

Adjacent 8-μm-thick sections were processed for the following immunostaining procedures (Table 1): (i) *Complete immunostaining procedure.* The immunoperoxidase method was applied (Sternberger et al. 1970). AFRU (dilution 1:1000) and an anti-bovine RF serum developed in rats, labeled RAFRU (R = rat, A = antiserum, FR = fiber of Reissner, U = urea: dilution 1:1000) were used as primary antisera. When AFRU was used, the secondary antibody was an anti-rabbit IgG raised in sheep (dilution 1:30), and the PAP complex (dilution 1:75) was prepared with an anti-peroxidase raised in rabbit. When RAFRU was employed as primary antiserum, an antirat IgG developed in rabbit (Sigma, dilution 1:50) and a rat PAP (dilution 1:50) were used. (ii) *Incomplete immunostaining procedure.* The sections were immunostained omitting the incubation in AFRU (Table 1).

The anti-rabbit IgG, anti-peroxidase raised in rabbits and rats, the rabbit-PAP and the rat-PAP were prepared in our laboratory

(Valdivia). For the immunostaining all antibodies were diluted in TRIS buffer, pH 7.8, containing 0.7% lambda carrageenan (Sigma). All incubations were performed at 22° C.

The complete immunostaining technique using AFRU was applied for the demonstration of both the secretory material of the SCO *and* the injected antibody, whereas the procedure using RAFRU was used to visualize *only* the secretory material. The incomplete immunostaining procedure demonstrated exclusively the injected antibody (see Fig. 35) (Table 2).

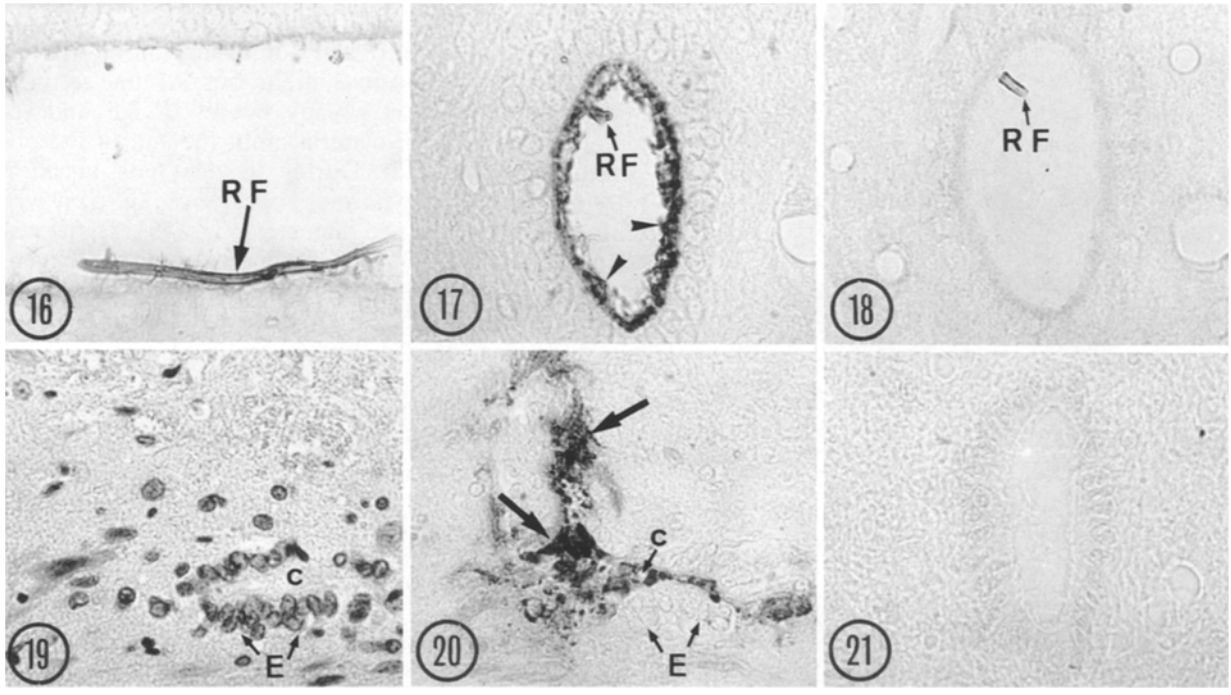
Ultrastructural immunocytochemistry

Three rats were injected with AFRU similarly to those of Group *1*; 20 min after perfusion ended the rats were anesthetized with ether and then vascularly perfused with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After 20 min of perfusion the brain was dissected out. A block containing the SCO was immersed in fresh fixative for 2 h. Postfixation was in 0.25% OsO_4 for 2 h. After dehydration in alcohols and acetone the blocks of tissue were embedded in butyl-methyl-methacrylate (Rodríguez et al. 1984a). Ultrathin sections mounted on nickel grids were sequentially incubated with: (i) anti-rabbit IgG developed in goat, 1:20 dilution, for 2 h; (ii) protein A-gold complexes for 1 h. All incubations were at 22° C in a moist chamber. Between incubations the sections were washed with phosphate-buffered saline, pH 7.4. Controls included similarly processed ultrathin sections but from SCO of untreated control rats.

Results

The incomplete immunostaining procedure applied to sections of the central nervous system was a very useful tool to demonstrate the location of the primary antibody contained in the antiserum against Reissner's fiber (AFRU), injected in vivo into the ventricular cerebrospinal fluid (CSF). The staining of adjacent sections with the complete immunoperoxidase method was used to establish whether the injected AFRU had been bound to the secretory material of the SCO (Fig. 35). A third adjacent section stained with the complete immunoperoxidase procedure, but using an anti-RF developed in rat (RAFRU) and a rat-PAP, demonstrated exclusively the SCO secretory material both in its intra- and extracellular location (Fig. 35). With respect to the SCO–RF complex the anti-RF serum developed in rabbits (AFRU) and that raised in rats (RAFRU) displayed the same staining properties.

The injected AFRU appeared in two distinct locations: (i) within the ventricular system it was exclusively bound to the released secretory material of the SCO, namely, the layer of secreted material covering the surface of the SCO, classified as pre-RF material by Rodríguez et al. (1986, 1987b) and the RF (Figs. 1–4); (ii)



Figs. 16–18. Rat 1 h after injection of AFRU into the CSF. **Fig. 16.** Sagittal section through upper cervical segments of the spinal cord. Incomplete immunostaining procedure. Reissner's fiber (*RF*) appears stained. × 370. **Fig. 17.** Same animal and staining as in previous figure. Transversal section through a low thoracic segment of the spinal cord. Reissner's fiber (*RF*) and wall of the central canal (*arrowheads*) appear immunoreactive. × 430. **Fig. 18.** Section adjacent to that of previous figure but immunostained with the complete immunostaining method using RAFRU. Only Reissner's fiber (*RF*) appears stained. × 430

Figs. 19, 20. Rat 4 h after injection of AFRU into the CSF. Adjacent sections through a lumbar segment of the spinal cord stained with hematoxylin-eosin (**Fig. 19**) and the complete immunostaining procedure using AFRU (**Fig. 20**). Clusters of immunoreactive material fill the central canal (*c*) and also appear to reach the adjacent neuropil (*arrow*). *E* ependyma. × 400

Fig. 21. Rat 1 d after administration of AFRU into the CSF. Transversal section through a cervical segment of the spinal cord. Complete immunostaining method using AFRU. Immunoreactive material and RF are not present in the central canal. × 420

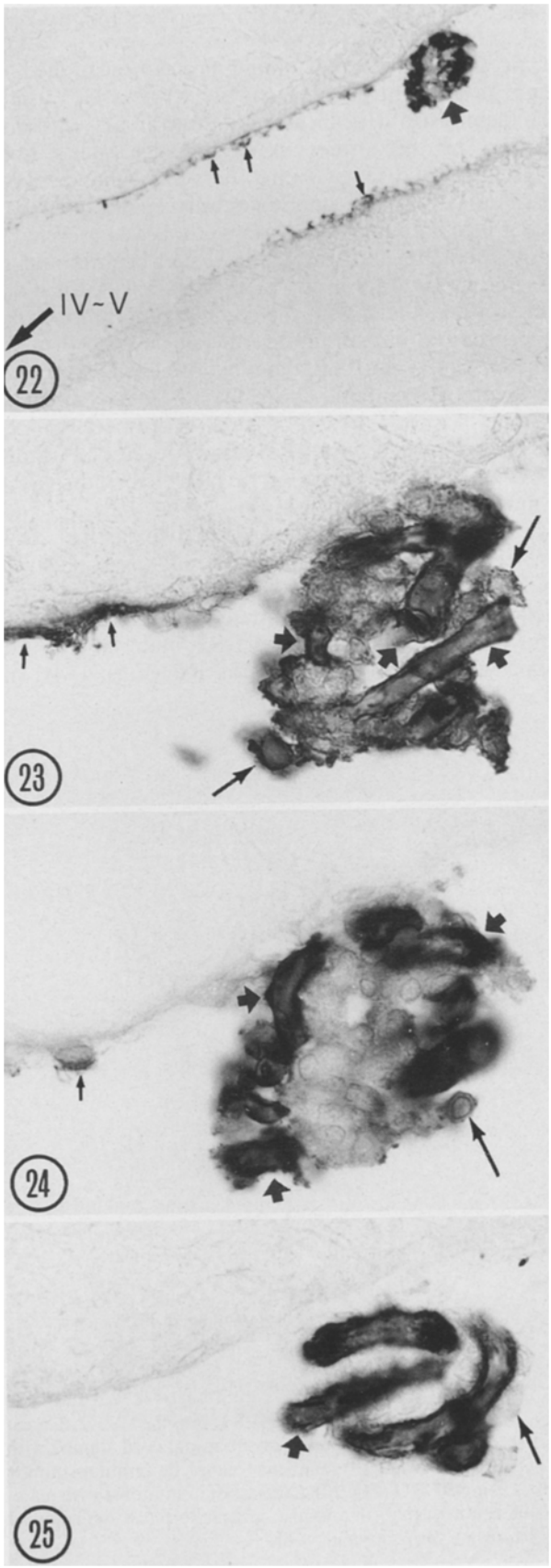


Fig. 22. Rat 3 days after injection of AFRU into the CSF. Sagittal section through the cavity connecting the Sylvian aqueduct with the fourth ventricle (IV–V). *Large arrow* indicates the flow of the CSF. Incomplete immunostaining procedure. Note immunoreactive material on the surface of the ventricular cavity (*small arrows*) and in a cluster of fragments of RF (*thick arrow*). × 180

within the subarachnoid space where the injected antibody appeared to be bound to the tissue layers bordering this space (Figs. 1, 2). The incomplete immunostaining procedure applied to ultrathin section allowed the ultrastructural localization of the injected antibody. It was confirmed that the latter was bound to RF, pre-RF and masses of secretory material located between the apical plasma membrane of the SCO cells, and the pre-RF (Figs. 5, 6). No label was found intracellularly.

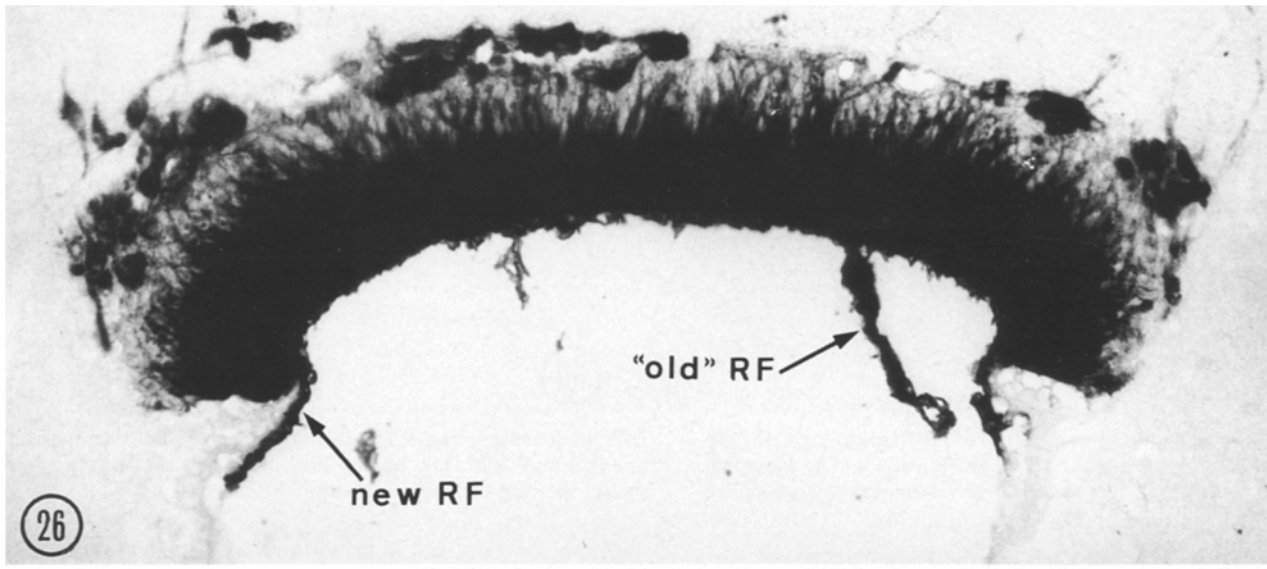
Events occurring at different time intervals after injection of AFRU into the CSF

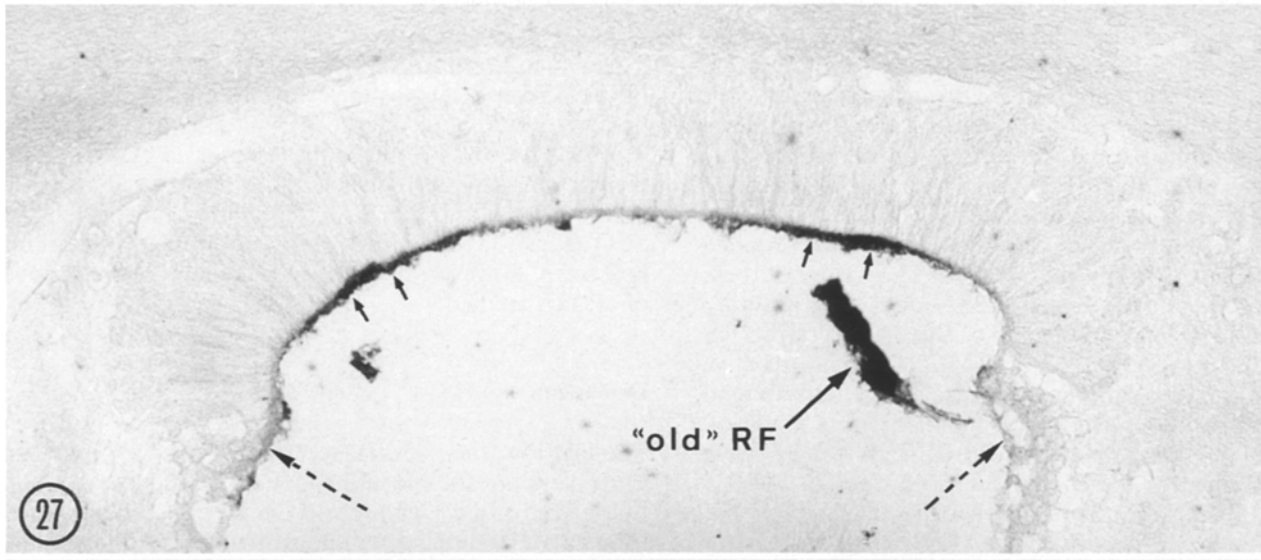
AFRU was injected slowly into the CSF (1 µl/min) over a period of 20 min. The time at which the perfusion was terminated was regarded as time zero. At this time point, AFRU had already reached and bound to the pre-RF and RF material (Figs. 7–9). Cross sections of RF showed that the antibody had penetrated the entire fiber (Fig. 9). At all post-injectional intervals studied up to day 1, the injected AFRU appeared bound to the pre-RF material, although the amount of the reaction product decreased progressively from 8 h post-injection onward (compare Figs. 1, 14, 27). Even 3 days after the injection of AFRU small surface areas of the SCO could be found where the antibody was fixed to the pre-RF material. In the subarachnoid space the injected antibody was detected up to 8 h after injection.

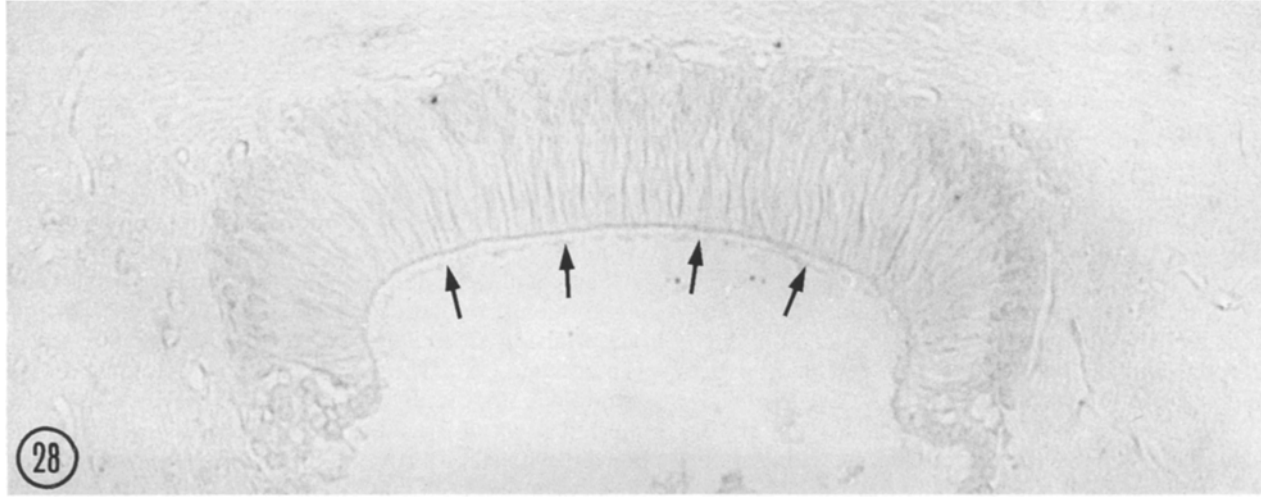
After the intraventricular administration of AFRU, RF underwent conspicuous alterations. At time zero the injected antibody was already bound to RF and remained bound to RF-material until the end of the observation period (8 d). During the first post-injection hour RF displayed a normal appearance, i.e. (i) it was uniform in structure and diameter (Figs. 16–18), (ii) was connected to the SCO, and (iii) was present in the Sylvian aqueduct, fourth ventricle and in the central canal of all segments of the spinal cord investigated (up to the lumbar level) (Figs 16–18). In this group of rats (1 h) the injected AFRU was detected in the central canal up to the lower thoracic segments. Within the central canal AFRU was bound to RF and to the luminal border of the ependymal cells (Fig. 17).

In all groups of rats investigated 4 h after injection, RF had detached from the SCO and appeared in the

Figs. 23–25. Adjacent sections through the same cluster of fragment of RF shown in **Fig. 22,** immunostained with the complete procedure using AFRU as primary antibody (**Fig. 23**), the incomplete procedure (**Fig. 24**), and the complete procedure using RAFRU as primary antibody (**Fig. 25**). Certain pieces of the fragmented RF are stained with all three methods (*thick short arrows*). The cells invading the clusters (*long full arrow*) and also the material covering the ventricular surface (*small arrows*) can be stained only by means of the first two methods (**Figs. 23, 24**). *Broken arrow* in **Fig. 25** points to unstained cells. **Figs. 23–25.** × 650

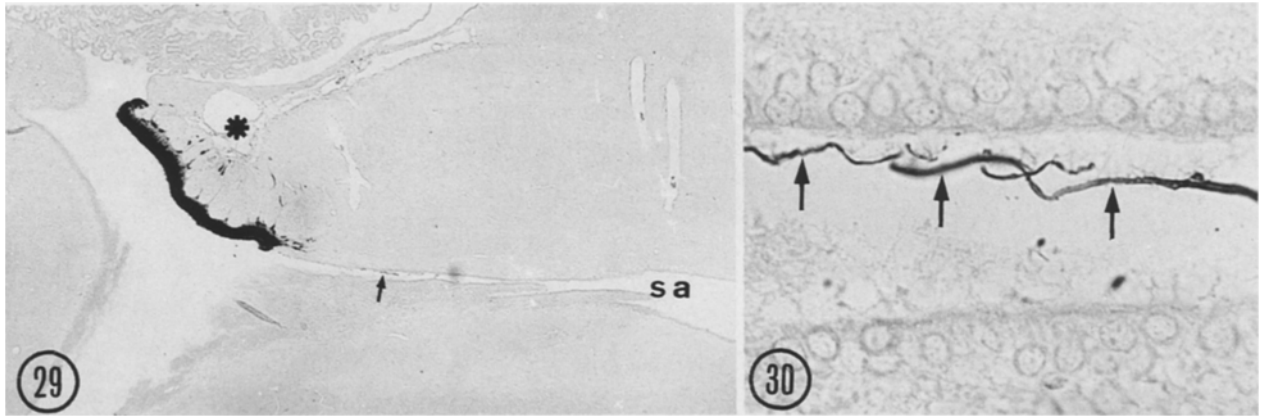






Figs. 26, 27. Rat 1 day after an intraventricular injection of AFRU. Adjacent frontal sections through the SCO immunostained after the use of the complete (**Fig. 26**) and incomplete (**Fig. 27**) procedures. An irregular fibrous structure is stained with both procedures (*"old" RF*). Only some of the pre-RF material is reactive with the incomplete procedure (*small arrows*). The newly formed, growing RF is reactive with the complete method (*new RF*, **Fig. 26**) but non-reactive with the incomplete method (*long broken arrows*, **Fig. 27**). × 220

Fig. 28. SCO from a rat 3 days after injection of AFRU into CSF. Incomplete immunostaining procedure. Pre-RF material not stained (*arrows*). × 220



Figs. 29, 30. Rat 8 days after injection of AFRU into the CSF. **Fig. 29.** Sagittal section through the SCO and aqueduct (*SA*). Complete immunostaining procedure using AFRU. *Arrow* newly growing RF within aqueduct, *asterisk* subarachnoid space free of immunoreactive material. × 27. **Fig. 30.** Detailed magnification from previous figure, showing the newly growing RF (*arrows*). × 600

form of reactive masses formed by twisted fragments of the disintegrated fiber extending along the Sylvian aqueduct and 4th ventricle (Figs. 15, 22–25). In the spinal cord RF-material appeared as irregular masses concentrated within the central canal, at the lumbar level (Figs. 19, 20). Part of the RF-material appeared to escape from the central canal (Fig. 20). The upper levels of the spinal cord lacked RF. In the 12-h, 1-day and 3-day groups the clusters of RF fragments became surrounded and subsequently infiltrated by cells. The nature of these cells has not yet been investigated. 3 days after injection of the antibody, the fragments of the disintegrating RF continued to be AFRU- and RAFRU-immunoreactive (Figs. 23–25) and still kept the injected antibody bound (positive immunoreaction with the incomplete method). The free cells surrounding the fragments of RF were weakly stained by the complete procedure using AFRU (Fig. 23) and also by the incomplete procedure (Figs. 24), but they were not reactive to RAFRU (Figs. 25).

Between day 1 and 8 after the injection of AFRU, RF or RF-material was missing from the central canal of the spinal cord (Fig. 21).

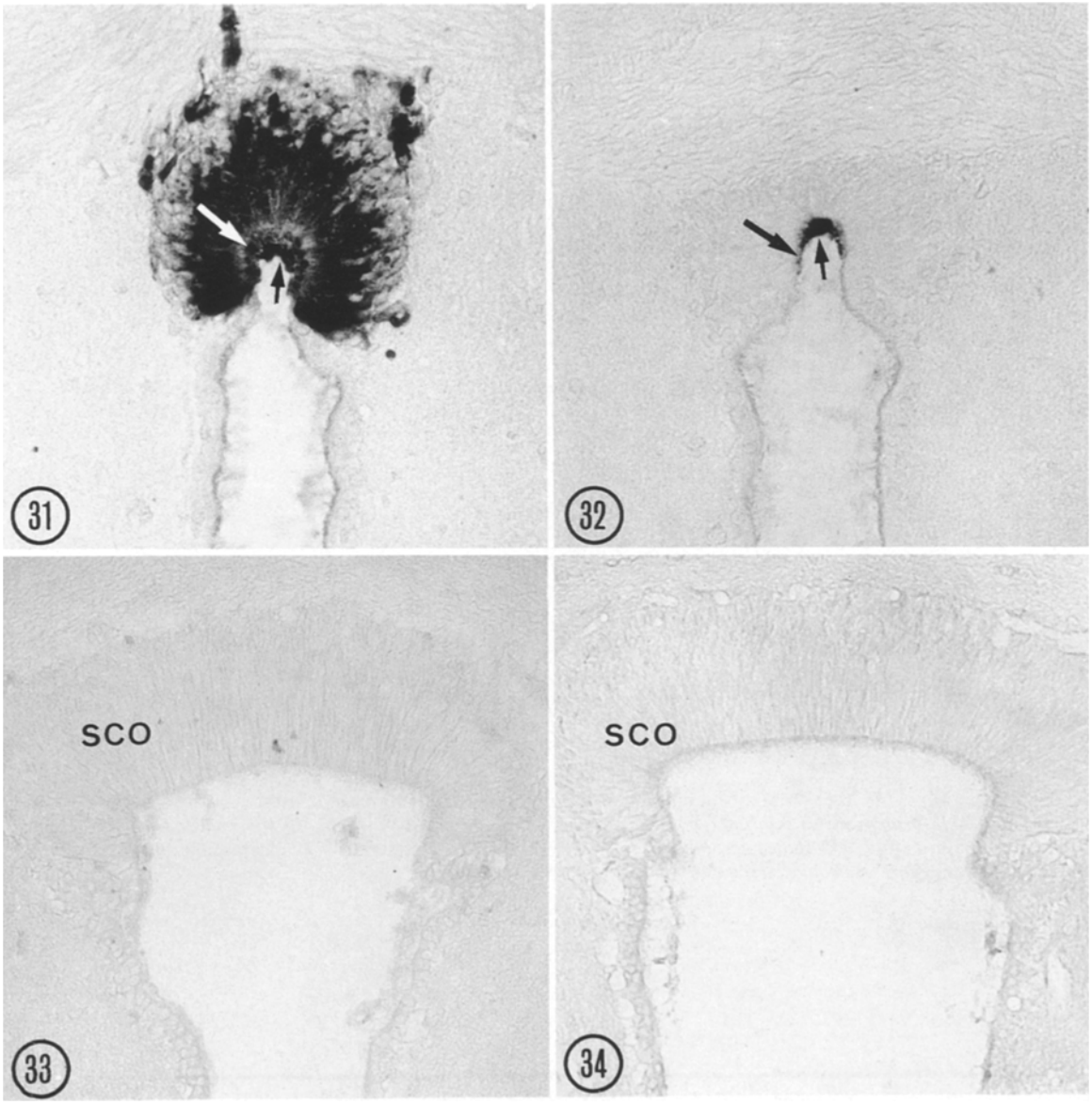
In the 1-day-group, a newly formed RF was seen to be growing from the surface of the SCO (Fig. 26). This fraction of the fiber had a regular appearance, was AFRU and RAFRU immunoreactive, but was not stained with the incomplete immunostaining procedure (Fig. 27). It was missing from the Sylvian aqueduct. This newly formed RF clearly contrasted with the appearance of the irregular fibrous structures that remained attached to the surface of the SCO and which were stained by the three methods, thus indicating that they were composed of RF-material-antibody complexes (Figs. 26, 27). The rats from the day-3 group showed changes similar to those of the day-1 group, the only difference being that in the former the pre-RF was virtually devoid of bound AFRU (Fig. 28) and the clusters formed by pieces of RF and infiltrating cells were scarce. At days 3 and 5 the newly formed RF was still missing from the aqueduct; but at day 8 it was found along the aqueduct (Figs. 29, 30), reaching the cephalic half of the fourth ventricle.

Control experiments

When an antiserum (ASO) against the secretory material of the bovine SCO was injected into the rat ventricle, it bound selectively to the pre-RF material and RF (Figs. 31, 32). In contrast, when anti-vasopressin raised in rabbit or rabbit IgG were injected into the rat CSF, neither antibody bound to the released secretory material of the SCO (Figs. 33, 34). ASO, anti-vasopressin and rabbit IgG were, however, detected in the subarachnoid space of all rats studied.

Discussion

The fact that the material secreted by the SCO into the ventricle is not soluble in the CSF, but condenses first on the surface of the organ (pre-RF material) and only then forms a RF, offered a unique situation that allowed the present experiment to be performed. Thus, an antibody raised against the secretory material of the SCO can be used for immunocytochemical visualization of the antigen (SCO secretion) in tissue sections in its original intracellular location and *also* in the CSF-compartment after it has been released into the ventricular cavity. This situation was an essential pre-requisite for the design of the present experiment. Since an antibody administered in vivo does not penetrate the plasma membrane of the cell (Alberts et al. 1983), it may only bind to the corresponding antigen if the latter is located either on the cell surface, in the intercellular space, or circulated in fluids such as blood and CSF. Thus, if an antibody raised against the secretory compounds of the SCO (AFRU, ASO) were injected in vivo, it would be expected to react with the pre-RF material and RF. This, indeed, was the case in the present experiment. The pre-RF- and RF-antibody complexes formed in vivo were resistant to the fixation and embedding procedures so that they could be visualized in tissue sections by the use of anti-rabbit IgG and rabbit-PAP (Fig. 35). Although for descriptive purposes this was regarded as an "incomplete immunocytochemi-



Figs. 31, 32. Adjacent cross sections through the SCO of a rat killed 20 min after an intraventricular injection of an antiserum against an extract of bovine SCO (ASO) raised in rabbits. **Fig. 31.** Complete immunostaining method using AFRU as primary antibody. **Fig. 32.** Incomplete staining using antirabbit IgG and rabbit-PAP. Only pre-RF material (*large arrow*) and RF (*small arrow*) are stained with both methods. × 130

Figs. 33, 34. Cross section through the SCO of rats 20 min after an intraventricular injection of anti-vasopressin serum raised in rabbits (**Fig. 33**), and a solution of purified rabbit IgG (**Fig. 34**). Both sections were stained with the incomplete procedure. In both cases the SCO (*SCO*) is not reactive. **Fig. 33.** × 85; **Fig. 34.** × 100

cal procedure", in principle it represents a regular immunoreaction with the only difference that the primary antibody (AFRU) had been administered in vivo.

The application of the three methods (summarized in Fig. 35) to adjacent sections allowed to differentiate among (i) free secretory material (free antigen), i.e., secretory material that did not react in vivo with the injected antibody; (ii) secretory material-AFRU complexes (antigen-antibody complex) formed in vivo; and (iii) free AFRU, namely, antibody not bound to antigen and thus circulating in the CSF or being absorbed by ependymal cells (Table 2). The fact that the pre-RF material and RF react with the antibody raised in rat (RAFRU) in spite of the fact that they had bound the injected AFRU (Fig. 35) indicates that not all epitopes of the secreted material react with the injected AFRU, or that AFRU and RAFRU do not react with the same epitopes although both demonstrate pre-RF material and RF.

A careful analysis of all the events occurring at different time intervals after a single injection of AFRU into the CSF leads to several important conclusions (Fig. 36):

1. The detection in the subarachnoid space of the

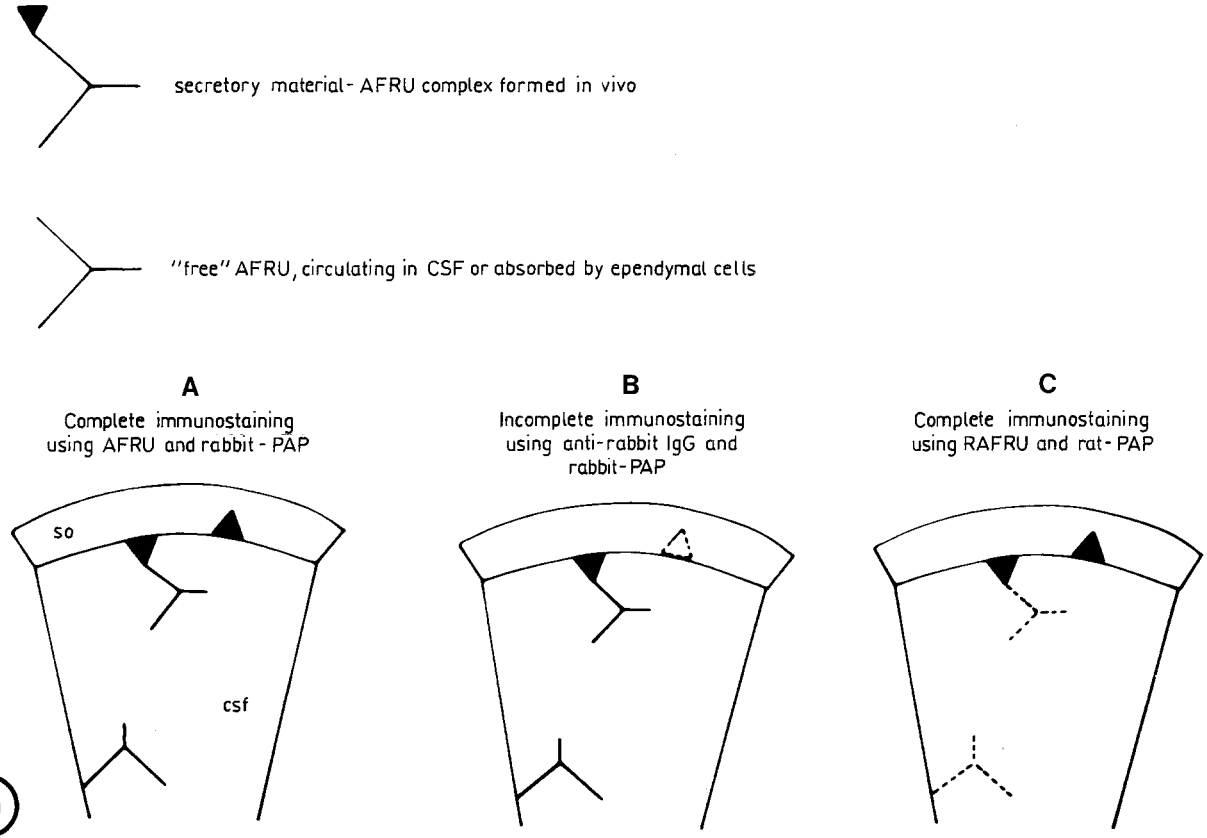


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Fig. 35. Schematic representation of the immunostaining procedures used. In A, B and C *solid triangles* and *solid IgG molecules* represent SCO secretory material and IgG molecules, which are immunoreactive. *Triangles* and *IgG molecules* indicated by *broken lines* represent secretory material and IgG molecules, which are not immunoreactive. *Triangles pointing upwards* represent intracellular secretory material. *SO* subcommissural organ cells; *CSF* cerebrospinal fluid

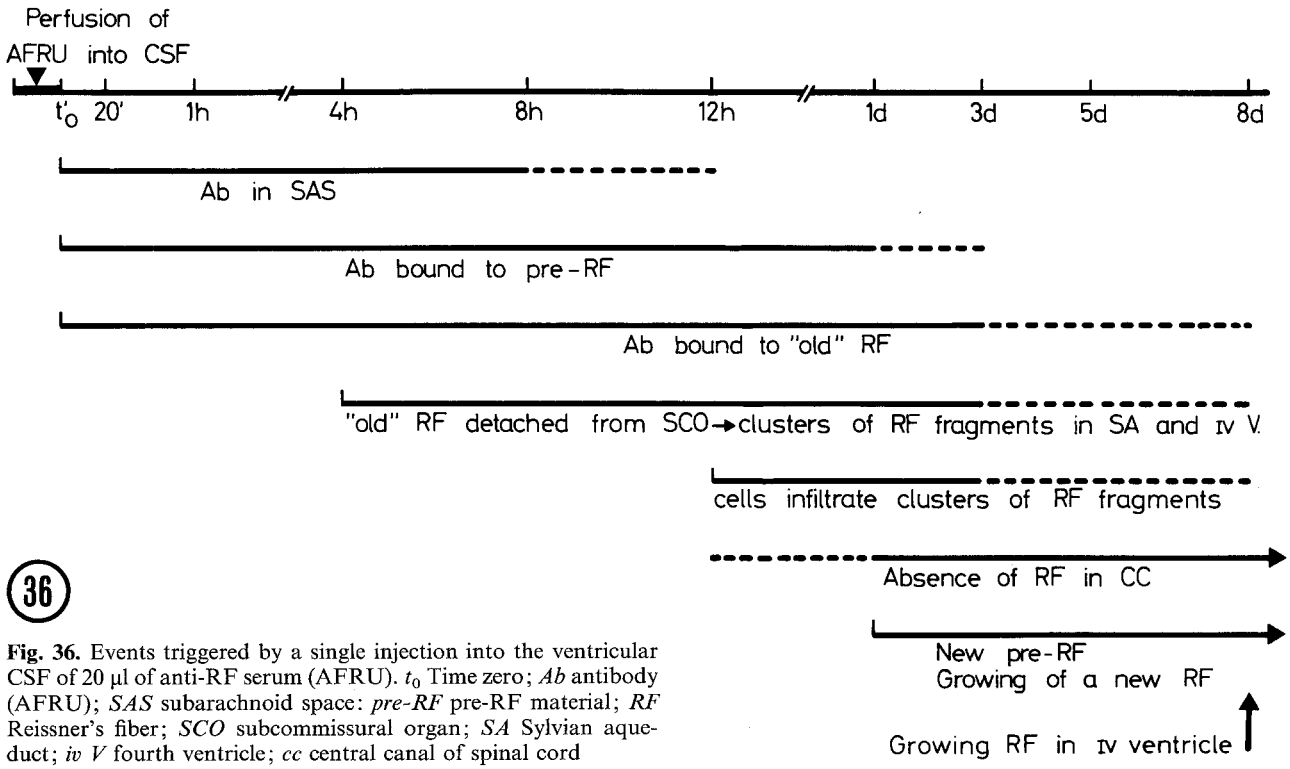

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Fig. 36. Events triggered by a single injection into the ventricular CSF of 20 µl of anti-RF serum (AFRU). t_0 Time zero; *Ab* antibody (AFRU); *SAS* subarachnoid space: *pre-RF* pre-RF material; *RF* Reissner's fiber; *SCO* subcommissural organ; *SA* Sylvian aqueduct; *iv V* fourth ventricle; *cc* central canal of spinal cord

injected antibodies between time zero and 8 h after injection and their absence from this space in rats killed 12 h or later after exposure to the injected antibody, most plausibly indicates that some time between the postinjection hours 8 and 12, the injected immunoglobulin had escaped or become cleared from the CSF. This correlates well with the fact that in those species where data are available, the complete renewal of the CSF occurs 4 to 5 times per day (Wood 1980; Cutler 1980).

2. If the injected antibody is no longer available 8 h after injection, the presence of AFRU bound to RF even at day 3 after its administration clearly indicates that the antigen-antibody complexes when formed within the CSF are very stable. It seems likely that these complexes are formed immediately after the injection of the antibody since RF was already labeled in the time-zero group.

3. The presence of antigen-antibody complexes in the pre-RF material is an important phenomenon and may become a useful tool to evaluate the rate of release of SCO-secretion. Indeed, the formation of these complexes involves (i) the released secretory material of the SCO before it condenses into RF and (ii) the antibodies available in the circulating ventricular CSF. While the latter are available (during the first 8 h post injection) the complexes in the pre-RF material are numerous. The progressive disappearance of these complexes starting at hour 8 is most probably due to the fact that the secretory material released after hour 8 post injection is unable to form complexes containing AFRU because the latter is no longer available. Since antigen-antibody complexes are very scarce or missing from the pre-RF material of rats killed at day 3, it may be concluded that during the first 3 days after a single injection of AFRU into the CSF the entire amount of secretory material assembled as pre-RF has been renewed.

4. The formation of a new RF at day 1 after administration of AFRU agrees well with the previous conclusion. Thus, when AFRU is no longer available in CSF, the material secreted by the SCO forms a new and apparently normal pre-RF and RF (Fig. 36). Considering that the RF of the rat and mouse grows at a rate of 10% of its length per day (Ermisch 1973, Herrera 1988), at day 3 after the injection the newly formed RF could be expected to occur at least at the level of the Sylvian aqueduct. However, this was not the case in our material. A possible explanation for this is that under the present experimental conditions the growth rate of RF is much slower than in the untreated rat, at least during the first days after the in vivo administration of AFRU. The fact that at day 5 RF is still missing from the aqueduct, and that 3 days later (day 8) it is already present in the fourth ventricle supports the assumption that during the first 5 postinjectional days, the growth rate of RF is lower than in the following days, when such a rate would reach expected values.

5. A single injection of AFRU into CSF leads to the detachment of RF from the SCO and its fragmentation. A plausible explanation for the detachment of RF is that the antibodies bound to the pre-RF material interfere with RF formation.

6. All RF fragments displayed antigen-antibody complexes. This indicates that fragmentation occurs along the whole length of RF, even at it most distal levels.

7. The rats injected with AFRU are devoid of RF in their spinal cord for at least 8 days (the longest investigated period).

In summary, it is concluded that a single injection of an antiserum against RF leads to an immunological blockade of RF formation. It seems most likely that the antibodies against the secretory glycoproteins of the subcommissural organ (AFRU), while available in the ventricular CSF, will immunoreact with these glycoproteins immediately after they are released into the CSF, thus forming antigen-antibody complexes. The formation of these complexes would, in turn, prevent the formation of RF by interfering with the assembly of the secretory glycoproteins. This effect is reversible. Repetitive injections of AFRU (every 8 or more days) might lead to a permanent blockade of RF formation.

The lack of binding of the anti-vasopressin serum and the rabbit IgG to the pre-RF material and RF demonstrates that the in-vivo binding of AFRU and ASO to the material secreted by the SCO is a specific antigen-antibody reaction.

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