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Immunohistochemical and histopathological studies of fixed rabies virus in goats

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ABSTRACT

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The purpose of this study was to systematically demonstrate and compare the pathological and immunohistochemical changes in goats which were infected by a fixed rabies virus that was used in vaccine production.

In the histopathological examinations, varying degrees of inflammatory, degenerative and necrotic changes were detected in the central nervous system. In the preparations stained by the immunoperoxidase (IP) method, intra- and/or extracellular viral antigens were observed on the cerebellum, cornu ammonis, thalamus, pons, nucleus caudatus, spinal cord, medulla oblongata, Gasserian ganglion, eye and retropharyngeal lymph nodes. In the preparations stained by the immunofluorescence (IF) method, intra- and/or extracellular viral antigens were seen in the same locations with the exception of the retropharyngeal lymph nodes. It was also observed that the antigens were qualitatively and quantitatively well stained with both methods. However, the visibility of antigens in the retropharyngeal lymph nodes and eye, and the facilities of applying made the IP method much more advantageous than the IF method.

Keywords: Histopathology, immunofluorescence, immunoperoxidase, rabies virus

INTRODUCTION

Rabies viruses include the street virus, the causative agent of rabies in humans and animals through natural transmission, and the fixed rabies virus, a laboratory-adapted form. The latter virus was developed by Pasteur with serial intracerebral passages of the street virus (Sullivan 1993). The virulence and incubation period of the fixed rabies virus are constant (Buxton & Fraser 1977; Jayakumar, Ramadass & Baghavan 1989). It is employed in many ways, such as in studying the replication of viruses and in the development of vaccines (Tordo & Kouknetzoff 1986; Consales, Valentini, Albas, Mendonca, Fuches, Soares & Pereira 1988).

For the diagnosis of naturally occurring rabies by histopathological examination, it is important to demonstrate the presence of Negri bodies in addition to the nonpurulent encephalomyelitis. However, Negri bodies are found in only 50–80 % of the cases (Goldwasser & Kissling 1958; Atanasiu, Dragonas, Tsiang & Harbi 1971; Koprowski 1973). Demonstration of the viral antigen with the use of immunohistochemical methods greatly increases the chances of diagnosing the disease. There is an 87–98 % possibility of diagnosing rabies by using the immunoperoxidase (IP) method and 87–100 % by the immunofluorescence (IF) method (Anjaria & Jhala 1985; Kotwal & Narayan 1985, 1987; Jayakumar *et al.* 1989; Zimmer, Weigand, Manz, Frost, Reinacher & Frese 1990). Since Negri bodies are not formed after inoculation of the animals with fixed rabies virus, it is impossible to detect infection on histopathological examination as in the case of street virus infection. However, IP

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and IF methods are employed to detect viral antigens in the tissue and organs of animals experimentally inoculated with the fixed rabies virus (Madhusudana & Tripathi 1990; Jackson & Park 1998).

The purpose of this study was to systematically demonstrate and compare the pathological and immunohistochemical changes in goats which were infected by a fixed rabies virus that was used in vaccine production.

MATERIALS AND METHODS

The examined materials were obtained from ten goats, each of which had been infected by the intracerebral inoculation of 0.2 mℓ of diluted Challenge Virus Standard (CVS) used for the production of a rabies vaccine by the Etlik Veterinary Control and Animal Diseases Research Institute in Turkey. These animals were slaughtered when they became agonized after the injection.

At postmortem examination tissue samples of each animal were taken from the cornu ammonis, nucleus caudatus, thalamus, pons, cerebellum, medulla oblongata, cervical spinal cord, Gasserian ganglion, parotid and submandibular salivary gland, retropharyngeal lymph node, vestibular region of the nose, an intact eye, skin around the ear and mouth, trachea, thymus, lung, heart, spleen, liver, kidney and adrenal gland. These samples were fixed in 10 % buffered formalin and embedded in paraffin wax. Sections were cut at 5 μm and stained with haematoxylin and eosin, and, if deemed necessary, with Periodic acid-Schiff and Luxol fast blue (Luna 1968). Fixed rabies virus antigens in the tissue were demonstrated by using polyclonal rabbit anti-nucleocapsid protein sera (obtained from the Rabies Centre of Expertise, OIE Reference Laboratory for Rabies, WHO Collaborating Centre, Animal Disease Research Institute, Canada) by means of a modified IP method (Strept-Avidin Biotin Peroxidase) and an indirect IF method (Heyderman 1979; Noorden & Polak 1983; Fekadu, Greer, Chandler & Sanderlin 1988; Vural 1997).

RESULTS

In the macroscopical examinations, the meningeal blood vessels were congested. In two cases, the retropharyngeal lymph nodes were swollen and appeared to be more moist than usual and dark brown in colour in cut surface.

In the histopathological examinations, many of the blood vessels in the central nervous system were hyperaemic and surrounded by lymphocytic cells in which there were small amounts of macrophages (Fig. 1A). Similar perivascular infiltrations were also observed in the meningeal blood vessels of the cerebral cortex, cerebellum, pons, thalamus and nucleus caudatus. Some neurons in these areas were also found to be degenerative or necrotic (Fig. 1B–D). The Nissl bodies in the neurons of the pons, cerebellum, medulla oblongata and spinal cord were diffusely distributed in an irregular, rough granular

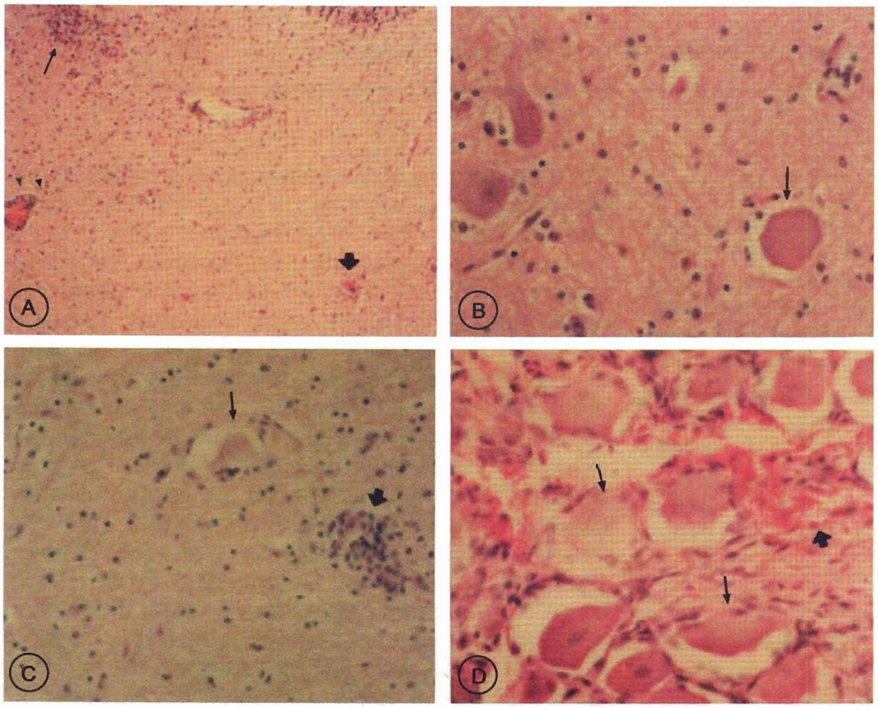

FIG. 1A Hyperaemia, perivascular infiltration (thin arrow) and neuronophagia (thick arrow) in the cerebellum

HE X100

FIG. 1B Neuronal necrosis (arrow) in the pons

HE X320

FIG. 1C Neuronal necrosis (arrow) and neuronophagia (thick arrow) in the thalamus

HE X200

FIG. 1D Neuronal necrosis (thin arrows) and haemorrhagia (thick arrow) in the Gasserian ganglion

HE X400

form or located at the periphery of the cytoplasm in the form of small granules. Some neurons of the medulla oblongata, pons and spinal cord of the animals were atrophic. Neuronophagia was apparent in the cornu ammonis, cerebellum (Fig. 1A), thalamus (Fig. 1C), pons, medulla oblongata, spinal cord and Gasserian ganglion. Babes' nodules were also observed in the cerebellum, thalamus and pons. Focally or diffusely distributed proliferation of glia cells was noticed in the cornu ammonis, cerebellum, thalamus, pons, medulla oblongata and spinal cord. Focal areas of demyelination in the cerebellum were observed in

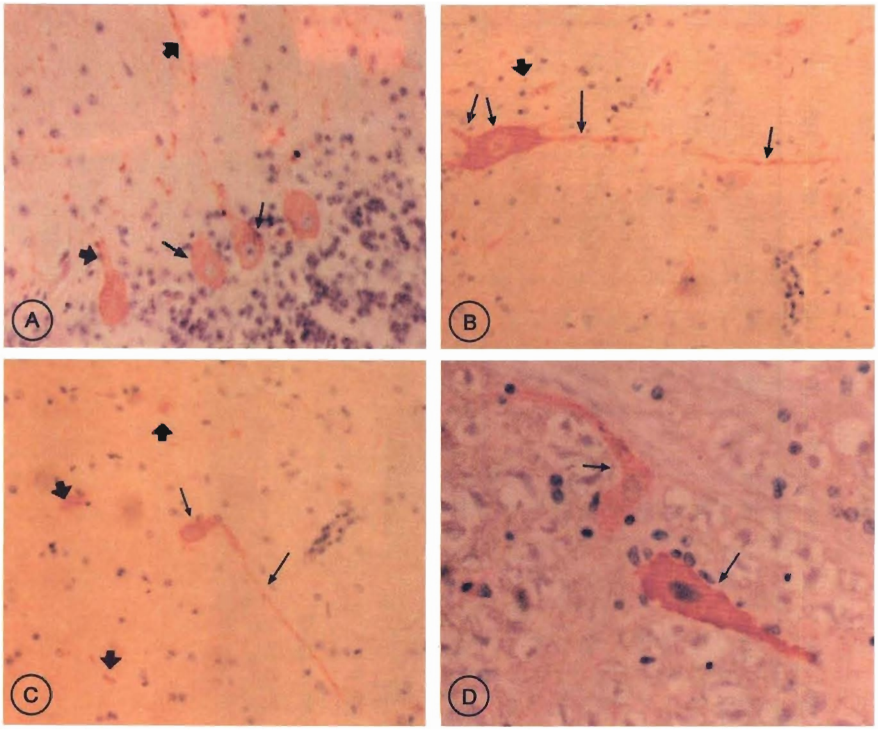

FIG. 2A Granular and/or diffuse IP-positive staining in Purkinje cells (thin arrows) and extensions (thick arrows)

X250

FIG. 2B IP-positive staining in a neuron and extensions (thin arrows) and neuropil (thick arrow) of the thalamus

X250

FIG. 2C IP-positive staining in a neuron and extensions (thin arrows) and neuropil (thick arrows) of the pons

X250

FIG. 2D IP-positive staining in the medulla oblongata (thin arrows)

X320

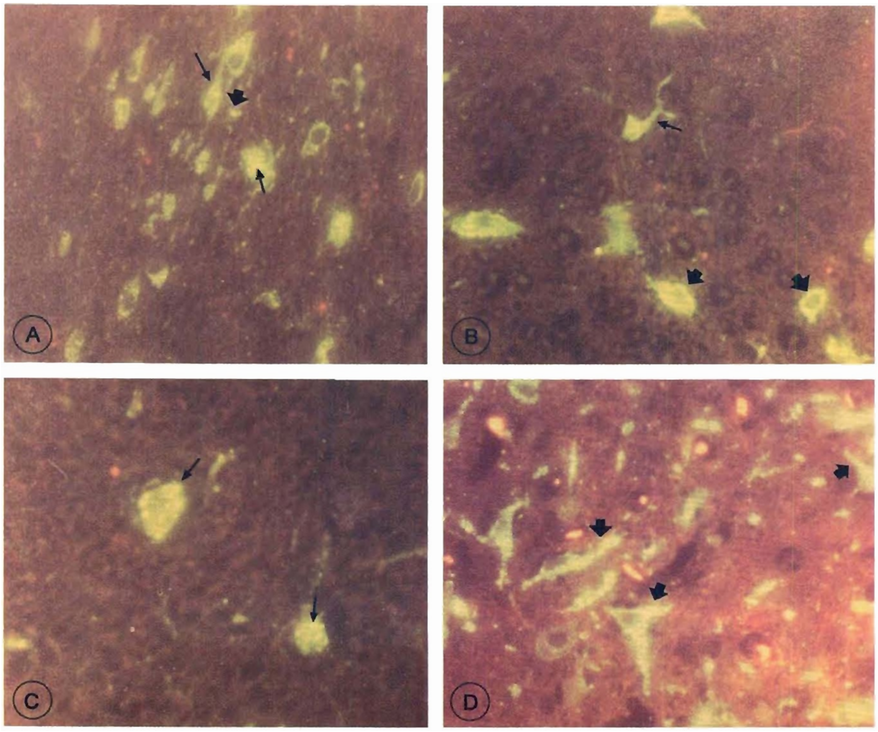

FIG. 3A IF-positive staining in neurons and extensions (thin arrows) and neuropil (thick arrow) of the cornu ammonis

X320

FIG. 3B IF-positive staining in a neuron (thick arrows) and extensions (thin arrows) of the pons

X320

FIG. 3C IF-positive staining in the thalamus (thin arrows)

X320

FIG. 3D IF-positive staining in the nucleus caudatus (thick arrows)

X320

TABLE 1 Results of the histopathological (HP), immunoperoxidase (IP) and immunofluorescence (IF) methods in different regions of the nervous system (NS)

Regions of the NS	Cornu ammonis			Cerebellum			Thalamus			Pons			Medulla oblongata			Spinal cord			Nucleus caudatus			Gasserian ganglion		
Examinations	HP	IP	IF	HP	IP	IF	HP	IP	IF	HP	IP	IF	HP	IP	IF	HP	IP	IF	HP	IP	IF	HP	IP	IF
Goat 1	+2	+1	+2	+3	+2	+3	+2	+2	+2	+3	+2	+1	+2	–	+2	+1	–	+1	–	+1	+2	+1	+1	–
Goat 2	+1	+1	+3	+1	+1	+1	+1	+2	+1	+2	+1	+1	+1	+1	+1	+1	–	+1	–	–	+1	–	–	–
Goat 3	+1	+2	+1	+2	+2	+2	+1	+3	+2	+2	+3	+2	+1	–	–	+1	–	–	–	–	–	–	–	–
Goat 4	+1	+1	+1	+1	+2	+2	+3	+2	+2	+2	+1	+2	+1	–	+1	+1	–	–	–	+1	–	+1	+1	+1
Goat 5	+2	+3	+2	+2	+2	+3	+2	+3	+1	+1	+2	+1	+2	+1	+1	+1	+1	+1	–	–	–	+1	+1	+2
Goat 6	+1	–	–	+3	–	–	+1	–	–	+2	–	–	+2	–	–	+2	–	–	–	–	–	–	–	–
Goat 7	+2	+1	+2	+3	+3	+3	+3	+1	+1	+3	+3	+1	+3	+2	+2	+3	+1	+1	–	+1	+2	+3	+1	+1
Goat 8	+2	+1	–	+3	+1	+2	+2	+1	+1	+3	+1	+1	+3	–	–	+2	–	–	+1	–	–	–	–	–
Goat 9	+3	+1	+1	+3	+1	+1	+3	+1	+2	+2	+1	+2	+2	+1	+1	+1	–	–	+1	–	–	–	–	–
Goat 10	+1	–	–	+2	–	–	+1	–	–	+1	–	–	+1	–	–	+1	–	–	–	–	–	–	–	–
Total	10	8	7	10	8	9	10	8	8	10	8	9	10	4	6	10	2	4	2	3	3	4	4	3

Grading marks: +1 = Light inflammation (HP), accumulations of viral antigen (IP, IF)
+2 = Mild inflammation (HP), accumulations of viral antigen (IP, IF)
+3 = Severe inflammation (HP), accumulations of viral antigen (IP, IF)
– = Not determined
Graded subjectively +1 to +3 according to amount of cellular infiltration and antigen present

some animals. Focal haemorrhages were noticed in the Gasserian ganglion (Fig. 1D), spinal cord and cornu ammonis. Perivascular oedema was also present in the pons of some animals. The extent of the varying degrees of nonpurulent encephalitis or meningoencephalitis observed in the animals is summarized in Table 1.

In the kidneys, some glomerular capillaries appeared to have taken the form of wire loops and others had undergone atrophy. The parietal membrane of Bowman capsules and the tubular basal membranes were thickened. Fibrosis was observed in some intertubular areas of the medullary regions and the areas surrounding the glomeruli where atrophy was present.

Sinus catarrh was seen in most of the retropharyngeal lymph nodes. The lymphoid follicles of the retropharyngeal lymph nodes and the thymus were hyperplastic. Lymphocytic cells and sparsely distributed macrophages were noticed in the vestibular region of the nose and trachea, particularly around some glands.

In the preparations stained by the IP method, viral antigens were observed (Table 1) in the Purkinje cells, cerebellar peduncle neurons and the stratum granulosum nerve cells of the cerebellum (Fig. 2A), pyramidal and cerebral cortex neurons of the cornu ammonis, thalamus (Fig. 2B), pons (Fig. 2C), nucleus caudatus, spinal cord, medulla oblongata (Fig. 2D), Gasserian ganglion, eye and retropharyngeal lymph nodes. They were seen as a fine dust or rough granules from brick-red to brown in colour in the perikaryons and extensions (axon and dendrites) of the neurons, glia cells and freely around them in the nervous system. They were also seen in the perivascular lymphocytic cells, endothelial cells and some ependymal cells of the pons and the medulla oblongata.

In IF stained preparations, the viral antigens were observed (Table 1) in the same regions of the central nervous system (Fig. 3A–D), the Gasserian ganglion and the eye. Viral antigens were revealed by the presence of glistening, yellow, mostly fine granules and some coarser particles. The granules in some sections were so big that they could be mistaken for inclusion bodies.

DISCUSSION

The necropsies of animals infected with fixed rabies viruses are generally reported not to reveal any pathological changes (Burkhart, Jervis & Koprowski 1950) or, as seen in this study, only hyperaemia in brain vessels was reported (Sinchaisri, Nagata, Yoshikawa, Kai & Yamanauchi 1992).

Perivascular mononuclear cell infiltration, glia cell proliferation, neuronal degeneration, neuronophagia and demyelination noticed in this study have been frequently reported as histopathological changes in the cerebral and cerebellar cortices, pons, spinal cord and Gasserian ganglion (Burkhart *et al.* 1950; Field 1951; Johnson 1965; Jackson & Park 1998). Inflammatory reactions reported in the meninges (Burkhart *et al.* 1950; Field 1951; Johnson 1965; Jackson & Park 1998) were also observed around the blood vessels on the meninges covering the cerebral cortex, cerebellum, thalamus, pons and nucleus caudatus in this study. In addition to these, perivascular oedema was seen in the meninges of the pons. Field (1951) has described the rough appearance of the Nissl granules in the neurons and stated that this is often encountered in the pons. Similar observations were made in the neurons of the pons, medulla oblongata, spinal cord and cerebellum in this study. As in other studies (Miyamoto & Matsumoto 1966; Sullivan 1993), neuronal degeneration and necrosis were results of only the fixed rabies virus infection.

The wire-loop formation of the glomerular blood vessels and the thickening of the parietal and tubular basal membranes in the kidneys of the vaccinated animals are thought to be the result of antigen-antibody complexes.

Fixed rabies viral antigens in IP preparations concentrate mostly in the cerebrum, cerebellum, pons, brain stem and the dorsal root ganglion of this region, and spinal and trigeminal nerves (Jackson, Reimer & Ludwin 1989; Jackson & Wunner 1991; Sinchaisri *et al.*1992; Jackson & Park 1998). In this study, IP positivity was encountered in the cornu ammonis, cerebellum, thalamus, pons, spinal cord, medulla oblongata, nucleus caudatus, Gasserian ganglion, eye and retropharyngeal lymph nodes. On the other hand, the antigens were mostly found in the grey matter besides in the white matter. This is similar to the findings in this study in which there were either relatively higher chances of finding antigens in this region, or else antigens are never found there (Jackson *et al.* 1989; Jackson & Wunner 1991).

With the IF preparations, the viral antigens were mostly present in the cerebrum, cerebellum, brain stem and the dorsal root ganglion of this region, spinal cord, trigeminal nerves, eye, heart, nasal mucosa, trachea, lung, kidneys, urinary bladder, adrenal gland, oral and stomach mucosae, the Averbach and Meissner plexus of the intestines, hair follicle and muscle (Correa-Giron, Allen & Sulkin 1970; Ravaioli, Palliola, Pestalozza, Granieri & Ciucnini 1970; Fischman & Schaeffer 1971; Johnson & Mercer 1964; Kucera, Doliva, Coulon & Flamand 1985; Coulon, Derbin, Kucera, Lafay, Prehaud & Flamand 1989; Tsiang, Lycke, Ceccaldi, Ermine & Hirardot 1989; Madhusudana & Tripathi 1990; Tsiang, Ceccaldi & Lycke 1991). In this study, the distribution of IF positivity in the central nervous system was almost similar. Although retropharyngeal lymph nodes have not been examined in the literature, no antigens were detected in this study.

Johnson & Mercer (1964) reported that viral antigens become localized only in the neurons, but not in glia, meningeal, ependymal or vascular cells and they tend to be concentrated in the neurons close to the ependymal cells and in the axons on the white matter; that IF positivity tends to increase, especially in the perikaryons and extensions of the Purkinje cells; and that viral antigens show irregular distribution on the cornu ammonis, cerebrum, brain stem, cerebellum and the anterior regions of the spinal cord. In this study, viral antigens were encountered not only in neurons, but also in extraneuronal regions close to the neurons and in some glia cells.

Although the same tissues that were used by Fischman & Schaeffer (1971), Madhusudana & Tripathi (1990), Jackson & Wunner (1991), were examined in this study, fixed rabies viral antigens were not observed in the IF and IP preparations except in the central nervous system, eye and retropharyngeal lymph nodes. In addition, contrary to the literature, viral antigens could not be traced in the IF and IP preparations in two of the cases necropsied despite inflammatory changes being present in all the animals. The variations obtained in this study are considered to be the result of the viral antigen being denatured at some stage, differences in inoculation methods and concentrations and strains of the viruses used. During the study it was found that organs from different animals yielded different results for the two methods. This is ascribed to practical errors. It was noticed in the qualitative and quantitative evaluation in the tissue and cells of the viral antigens.

Tsiang, Koulakoff, Bizzini & Berwald-Netter (1983) in IF examinations of neuroblastoma and dorsal root ganglion cell cultures infected with CVS, reported the finding of one to two cytoplasmic inclusions in each cell. However, Sinchaisri *et al.* (1992) could not detect inclusion bodies which they attributed either to the fact that the animals had died before the viral antigens could accumulate in sufficient quantity to form inclusion bodies, or to the experimental conditions that they used. Although inclusion bodies were not seen in this study, it could not be determined whether or not the large granules observed in the IP and IF preparations were inclusion bodies.

In the histopathological examinations done during this study, varying degrees of nonpurulent encephalitis or meningoencephalitis were observed, but these can also be caused by many other viral diseases. For doing a differentiative diagnosis the immunohistochemical examinations had to be done. It is therefore shown that the fixed rabies virus antigen in formalin-fixed and paraffin-embedded tissue sections are seen clearly by using the IP and IF methods in this study. Whereas the IF method requires a fresh preparation of the procedure each time as well as the use of fluorescent microscopy, the IP method is used with fixed specimens and requires a standard microscope. This has practical implications for the laboratories. All the IP preparations are permanent, which implies that they can be stored and reexamined if required. The antigens were well stained by both methods but were qualitatively and quantitatively superior in the IF method. However, the IP method better demonstrated the viral antigens in tissues such as the retropharyngeal lymph nodes and eye.

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