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Canine Distemper Virus-induced Glial Cell Changes in vitro*

A. Zurbriggen and M. Vandevelde

Institute of Comparative Neurology, University of Berne, P.O. Box 2735, CH-3001 Berne, Switzerland

Summary. In vitro studies on glial cell changes in canine distemper virus (CDV) infection could be useful for the understanding of the pathogenesis of demyelination in vivo in this disease.

Mixed glial cell cultures derived from neonatal mice and dogs were infected with CDV and examined using immunocytochemical techniques demonstrating specific oligodendroglial and astroglial cell markers. Astrocytic changes were similar in both murine and canine cultures and consisted of loss of processes, cell fusion, and cell necrosis. Marked oligodendroglial lesions were apparent in the canine brain cultures and were characterized by focal perikaryal protrusions, swelling and loss of cell processes, and cell necrosis. Fusion between oligodendrocytes was not observed. Fusion between astrocytes and oligodendrocytes could not be documented with double labeling techniques. In contrast to the canine cultures, murine oligodendrocytes remained relatively unaffected by the infection. These findings were discussed with respect to cell pathology and mechanisms of demyelination in vivo. The exact nature of the canine oligodendroglial lesions in vitro needs to be studied in further experiments.

Key words: Canine distemper virus – Brain tissue culture – Astrocyte – Oligodendrocyte – Demyelination

Introduction

Demyelination in canine distemper virus (CDV) infection is an animal model for paramyxovirus-associated neurologic disease in man (Koestner 1975). The pathogenesis of demyelination in distemper is uncertain. Recent studies have indicated that the initial myelin lesion in distemper is directly virus-induced (Summers et al. 1979; Vandevelde et al. 1982; Higgins et al. 1982). Lytic infection of oligodendrocytes as, e.g., in mouse corona virus infection (Dal Canto and Rabinowitz 1982) is not apparent in distemper. Astrocytes rather than oligodendrocytes appear to be the target of CDV (Raine 1976: Summers et al. 1979). Cell fusion between astrocytes has been documented in distemper and it has been proposed but not shown that oligodendroglial membranes may also be involved in this fusion process which could then lead to demyelination (Summers et al. 1979). All these results have been derived from in vivo experiments in dogs. Attempts to induce demyelination with CDV in more suitable animals, such as mice, have failed although this virus causes encephalitis and death in this species (Lyons et al. 1980; Gilden et al. 1981).

Useful information on CDV-induced glial cell pathology could be obtained from tissue culture studies. It has been shown that CDV infection prevented myelin formation or resulted in myelin degeneration in canine organotypic cerebellar explant cultures (Storts et al. 1968). Although changes, such as cell fusion and viral inclusion bodies, were described, the mechanism of myelin damage was not clear from this elegant study which was conducted before methods of specific glial cell identification were available. In recent years relatively simple brain tissue culture systems have been developed in which immunocytochemical methods allow unequivocal specific identification of astrocytes and oligodendrocytes (Wiesmann et al. 1975; Bologa-Sandru et al. 1981; Schnitzer and Schachner 1981). The aim of the study reported here was to use such mixed glial cell cultures to examine the specific effects of CDV on astrocytes and oligodendrocytes in vitro. Since CDV causes demyelination in the dog but not in the mouse, mixed glial cell cultures derived from each species were used and the results compared.

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Offprint requests to: M. Vandevelde, DVM (address see above)

Material and Methods

Animals

Eight newborn pups from a closed colony of Beagle dogs (12 h old or less) were a kind gift of Ciba-Geigy AG, Basel, Switzerland.

Five litters of newborn mice were derived from commercially available NMRI mice (Dr. Ivanovas, Kißlegg, FRG).

Brain Tissue Culture

The brains of the newborn mice and the cerebellum of the dogs were aseptically removed immediately after death, immersed in the medium, and mechanically dissociated by chopping and repeated aspiration in 10-ml plastic pipettes and Pasteur pipettes as described (Wiesmann et al. 1975; Zurbriggen et al. 1983). The cells were seeded in a concentration of 1.5×10^6 cells/ml in 10-cm plastic Petri dishes containing 10 ml DMEM with 10% fetal calf serum, 4.4 g/l D(+)-glucose, 2.2 g/l $NaHCO_3$, 250,000 U/l penicilin and 100 µg/l streptomycin. Eight 18×18 mm glass coverslips were attached to the bottom of the Petri dish with silicon grease. The cultures were kept at 37° C in a 5% CO_2, H_2O saturated atmosphere. They were left for 4 days after seeding; afterward the medium was changed every 2 days. Four consecutive experiments were carried out in each species. For each experiment 12 Petri dishes were prepared; half of these were infected, the other half served as uninfected controls.

Virus

Onderstepoort CDV, a tissue culture adapted CDV strain (Appel and Gillespie 1972) was a kind gift of Dr. U. Kihm, Vaccine Institute Basel, Switzerland, and was propagated in Vero cells. The virus was then passaged through newborn mice by intracerebral (i.c.) inoculation as described previously (Lyons et al. 1980). After the fifth passage, the virus was shown to kill newborn and 10-day-old mice within 48–72 h after i.c. inoculation. The mouse-adapted virus still replicated very well in Vero cells with a characteristic CPE (syncytium formation) (Appel and Gillespie 1972) and in primary brain cell cultures derived from mice and dogs. The virus titer of the material derived from the final mouse passage was determined to be 5×10^5 $TCID_{50}$ according to established procedures (Cunningham 1973). This material was prepared by disintegrating infected brain tissue in medium followed by centrifugation to remove cell debris. It was stored in small aliquots at −70° C until used.

Infection

Cultures were infected as soon as they reached the age at which they contained both astrocytes and oligodendrocytes in sufficient numbers (in dogs between 6 and 8 days after seeding, in mice between 9 and 10 days). Five to 50 $TCID_{50}$ CDV were used per milliliter of tissue culture medium. The cultures were covered with a small amount of virus-containing medium and placed in the incubator. After a period of 1 h during which virus absorption was allowed to take place more medium was added. Cultures were harvested at 24, 48, 72, and 96 h after infection. For every infected culture an uninfected one, derived from the same tissue culture batch, was also collected at the same time.

Fixation and Staining

Cultures were briefly rinsed with Hanks BSS and treated with phosphate-buffered formalin, Triton-X, and acetone as described (Bologa-Sandru et al. 1981). Coverslips were removed from the Petri dishes and stained with HE. Other coverslips were used for immunocytochemical detection of CDV, glial fibrillary acidic protein (cell marker for astrocytes), myelin basic protein and myelin-associated glycoprotein (both cell markers for oligodendrocytes). The immunocytochemical method used was the unlabeled antibody-PAP technique essentially as described by Sternberger (1979). Highly diluted primary antisera were left on the cultures for 48–72 h at 4° C. Selected cultures were used for double labeling experiments in which both astrocytes and oligodendrocytes were simultaneously visualized with two different chromogens. For double labeling two consecutive PAP procedures were carried out according to the technique of Sternberger and Joseph (1979) in which DAB (brown) was the first chromogen and alpha-chloronaphthol (blue) the second one. For better resolution of the alpha-chloronaphthol reaction product, the double bridging PAP method of Vacca et al. (1980) was used for the second antibody sequence. All coverslips were mounted on glass slides and examined with conventional and phase contrast microscopy.

Antisera

Canine anti-CDV antibody (Behringwerke AG, Marburg, FRG) and human anti-measles virus antibody (S.B. Bern, Switzerland) were used for demonstration of CDV.

Rabbit anti-GFAP (Dakopats, Copenhagen, Denmark) was used to demonstrate astrocytes. Rabbit antihuman MBP was a kind gift of Prof. Herschkowitz (Pediatric Hospital, University of Berne, Berne, Switzerland) and was used to demonstrate murine as well as canine oligodendrocytes. Canine oligodendrocytes were also stained immunocytochemically with a human serum containing monoclonal anti-MAG. This serum was derived from a patient with polyneuropathy and was shown to bind to canine but not to murine MAG in a previous study (Steck et al. 1983).

Results

Normal Uninfected Cultures

The canine cultures contained large clusters of astrocytes most of which had the characteristic GFAP+ pointed cell processes (Fig. 3A), and focal accumulations of MAG+ oligodendrocytes superimposed on the other cell types. The oligodendrocytes had their characteristic morphology with small angular perikarya and several long-branching processes (Fig. 4A, C). During the further development of the culture, increasing numbers of oligodendrocytes also expressed MBP. Details on the development and characterization of canine-dissociated brain cultures have been reported elsewhere (Zurbriggen et al. 1983). The murine cultures have been characterized in several publications (Wiesmann et al. 1975; Bologa-Sandru et al. 1981; Schnitzer and Schachner 1981). They also contained large clusters of astrocytes and MBP containing oligodendrocytes which were found on the surface of the monolayer. The MBP+ cells also had a tendency to occur in small clusters distributed throughout the culture (Fig. 2C).

Murine Cultures Infected with CDV

Twenty-four hours after infection a clear cytopathogenic effect (CPE) was detected in many areas of the culture. It consisted of small plaques in which the monolayer had acquired a reticulated appearance

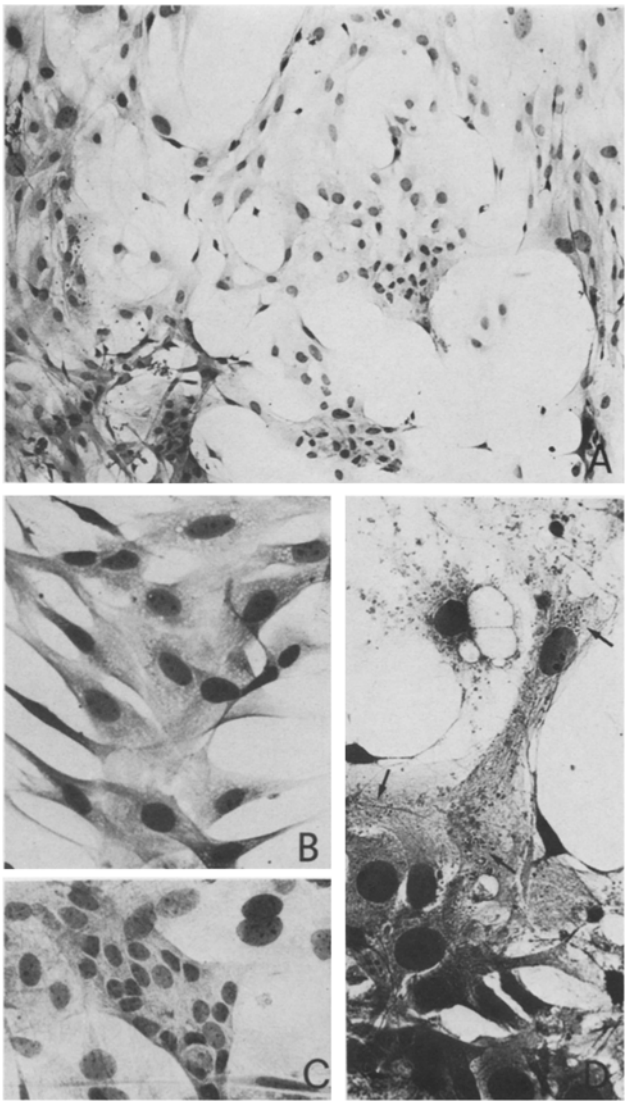

Fig. 1. A Murine brain culture 24 h p.i.; plaque formation. HE, × 28. **B** Canine brain culture 48 h p.i.; fused cells at the edge of a plaque. HE, × 178. **C** Murine brain culture 24 h p.i.; syncytium formation. HE, × 178. **D** Murine brain culture 48 h p.i.; cell necrosis in a plaque. Large number of small intracytoplasmic inclusion bodies (*arrows*). HE, × 280

(Fig. 1 A). Many cells in and around the plaques appeared to be enlarged, had decreased staining intensity of their cytoplasm, which contained many small inclusion bodies (Fig. 1 D). In many areas, multinucleated cells were seen (Fig. 1 B, C), apparently as a result of cell fusion, which is a typical feature of morbillivirus infection (Appel and Gillespie 1972). Several cells were undergoing necrosis. The plaque formation coincided with areas of intense viral replication as seen on immunocytochemical preparations for demonstration of viral antigen. Astrocytes were clearly involved in the plaque formation with loss of their typical pointed cell processes and enlargement of their perikarya which often contained only small amounts of GFAP (Fig. 2 A). They were also clearly involved in the cell fusion process (Fig. 2 B). Such astrocytic syncytia contained many small inclusion bodies as seen with phase contrast microscopy. The MBP + oligodendrocytes in the culture did not appear to be affected by the destructive process. As compared with control cultures, these oligodendrocytes – whether in the plaque areas or in their vicinity – did not show morphologic changes. Forty-eight hours after infection CPE had progressed with several small plaques merging together. The cellular changes were similar as at 24 h after infection with cell necrosis and cell debris becoming apparent. Viral infection was judged by the presence of inclusion bodies and viral antigen had become widespread in the culture. Seventy-two hours after infection the CPE had strongly increased with destruction of one half to two thirds of the culture. Nearly all remaining cells contained viral antigen. Most remaining astrocytes had lost their typical structure with pointed cell processes and became rounded with loss of clear borders between them and expression of various amounts of GFAP. Oligodendrocytes were reduced in number as compared to the controls since their supporting monolayer (as described, oligodendrocytes grow on top of other cells) had completely disappeared in large areas. However, many normal appearing MBP + cells had survived in those areas were the monolayer was still more or less present (Fig. 2 D). At 96 h, most of the culture was destroyed, with only small strands of monolayer left. Normal astrocytes were no more available, only a few weakly GFAP + cells were seen. Surprisingly, several normal appearing MBP + oligodendrocytes were still present.

Canine Cultures

Twenty-four hours after infection very little CPE was seen. After 48 h several small plaques appeared, the morphology of which was very similar to the lesions in the murine cultures including syncytium formation, inclusion bodies, and cell necrosis. Astroglial changes were also similar with loss of cell processes, perikaryal enlargement, and fusion of adjacent cells (Fig. 3 B, C). MAG + oligodendrocytes appeared to be reduced in number as compared to control cultures. Very striking were the morphological changes in these cells. Large numbers of oligodendrocytes, not only in the vicinity of plaque areas but throughout the culture, had lost their normal sharply defined cell contours. Such cells had irregularly shaped cytoplasmic protrusions which occurred at the level of the perikaryon extending into the cell processes (Fig. 4 B). Only sporadic MBP + oligodendrocytes were found as compared to the control

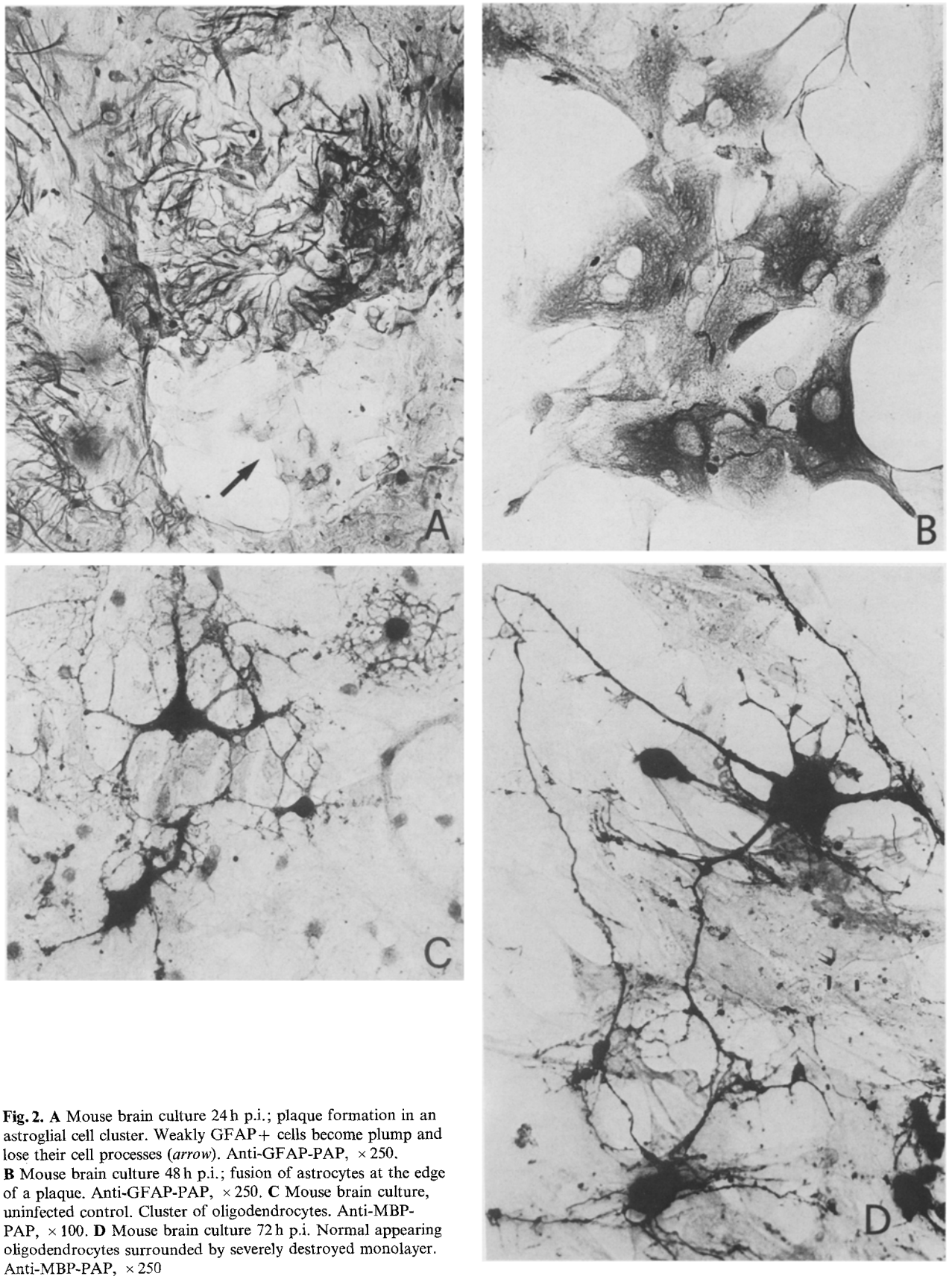

Fig. 2. A Mouse brain culture 24 h p.i.; plaque formation in an astroglial cell cluster. Weakly GFAP+ cells become plump and lose their cell processes (*arrow*). Anti-GFAP-PAP, ×250. **B** Mouse brain culture 48 h p.i.; fusion of astrocytes at the edge of a plaque. Anti-GFAP-PAP, ×250. **C** Mouse brain culture, uninfected control. Cluster of oligodendrocytes. Anti-MBP-PAP, ×100. **D** Mouse brain culture 72 h p.i. Normal appearing oligodendrocytes surrounded by severely destroyed monolayer. Anti-MBP-PAP, ×250

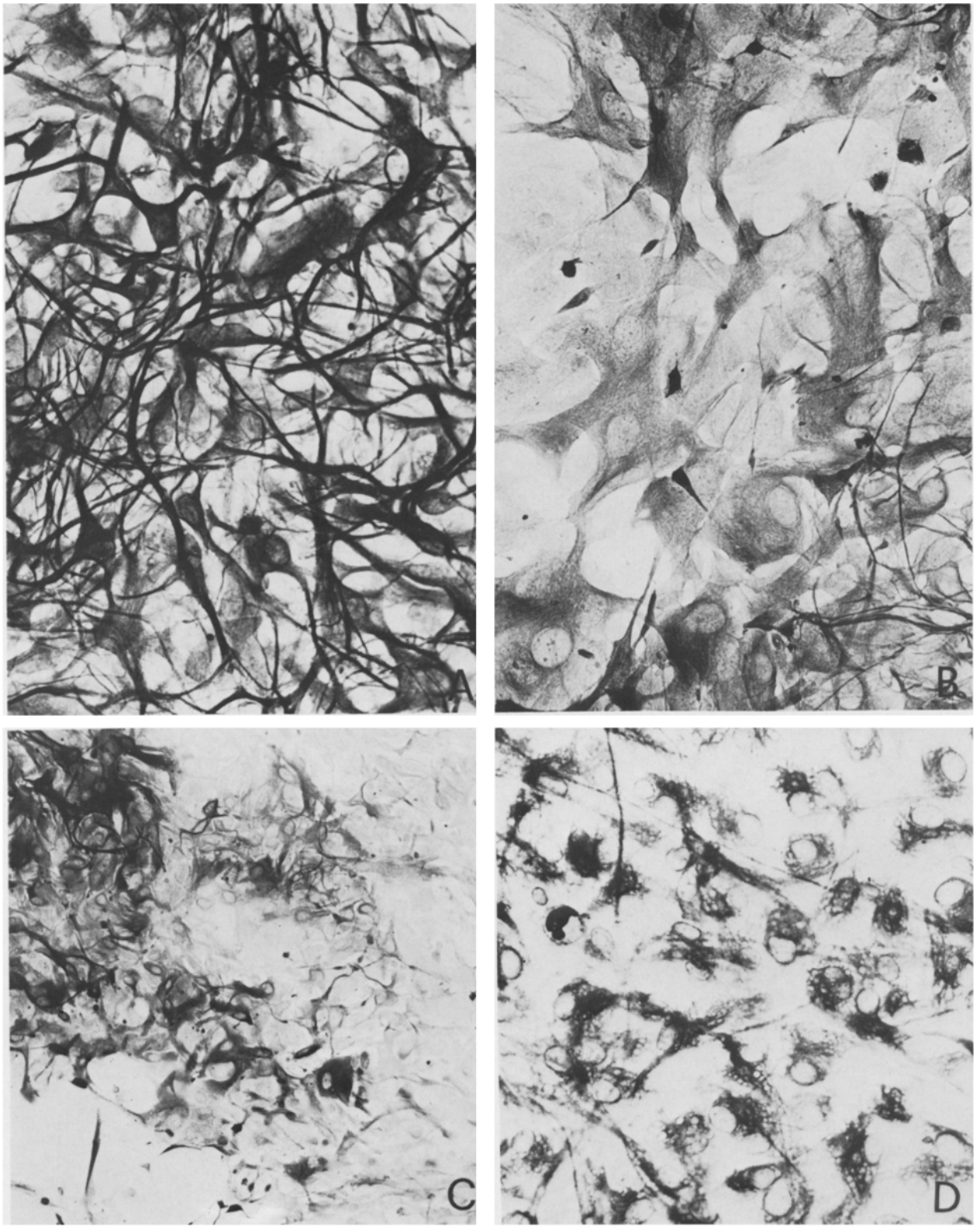

Fig. 3. A Canine brain tissue culture; normal control, typical appearance of astrocytes with GFAP-containing processes. Anti-GFAP-PAP, × 250. **B** Canine brain tissue culture 48 h p.i.; astrocytes in plaque area have lost their processes, become large and plump and fuse with each other. Anti-GFAP-PAP, × 250. **C** Canine brain tissue culture 72 h p.i.; widespread astroglial changes: loss of pointed cell processes; blurred cell borders. Anti-GFAP-PAP, × 100. **D** Canine brain tissue culture 72 h p.i; cluster of small rounded vacuolated astrocytes. Anti-GFAP-PAP, × 250

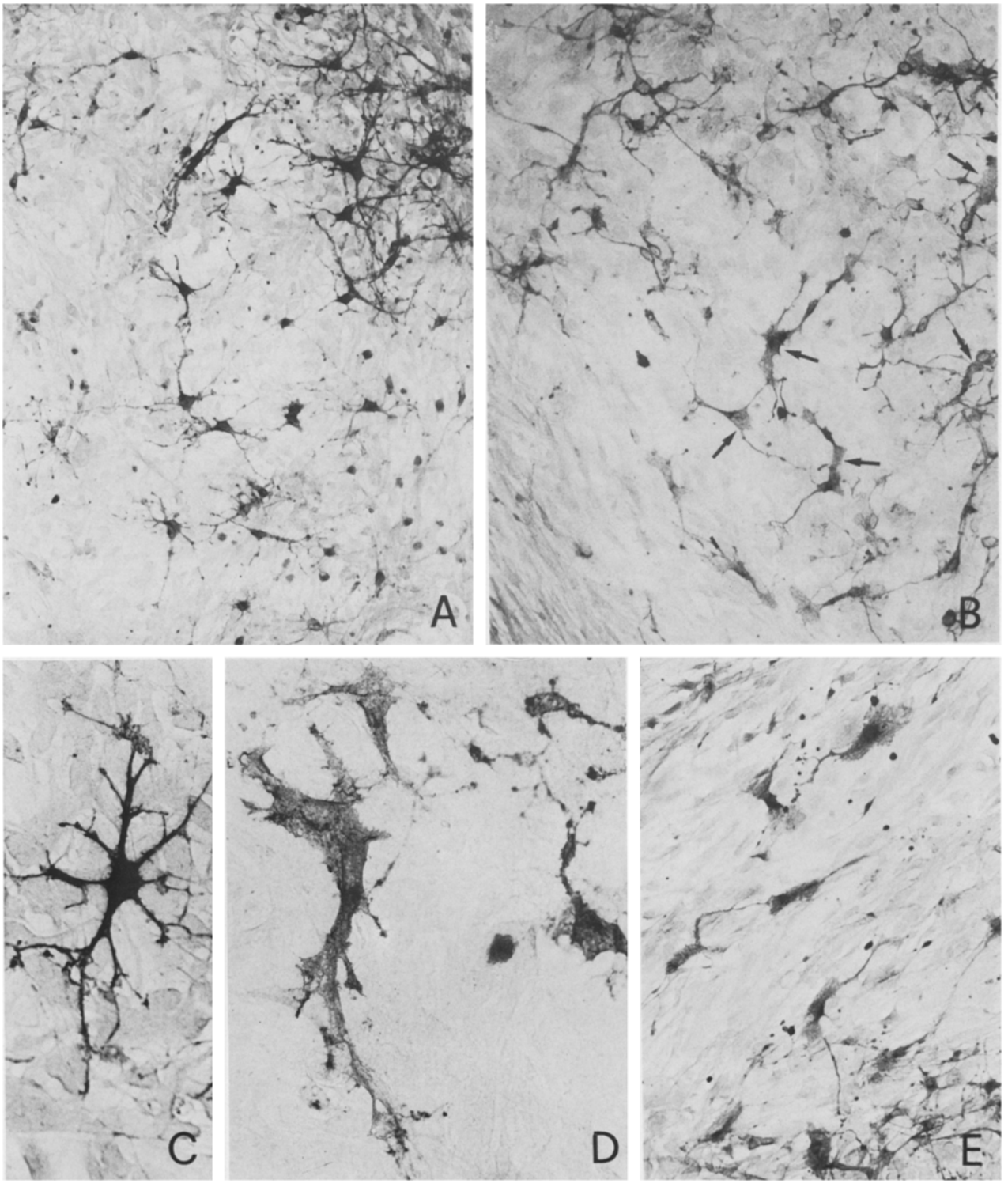

Fig. 4. A Canine brain tissue culture, normal control. Oligodendrocytes with angular perikarya and several fine branching processes on the surface of the monolayer. Anti-MAG-PAP, × 100. **B** Canine brain tissue culture 48 h p.i. Most oligodendrocytes with irregular distentions of perikarya and cell processes (several abnormal cells depicted by *arrows*). Anti-MAG-PAP, × 100. **C** Canine brain tissue, normal control. Typical oligodendrocyte with sharply defined cell borders. Anti-MAG-PAP, × 250. **D** Canine brain tissue culture 72 h p.i. Oligodendrocyte with swollen perikaryon and processes. Anti-MAG-PAP, × 250. **E** Canine brain tissue culture, 96 h p.i. Remaining oligodendrocytes are reduced to cell ghosts. Anti-MAG-PAP, × 250

cultures. At 72 h, CPE had progressed considerably: astroglial changes were very prominent: in large clusters of astrocytes, cells had lost their processes and were small, round and vacuolated (Fig. 3D). Nearly all MAG+ oligodendrocytes in the culture had undergone the above described change and many cells had lost their long branching cell processes (Fig. 4D). Only very few MBP+ oligodendrocytes were noted as compared to the controls. At 96 h, most of the culture was destroyed. Normal astrocytes were no longer present. Most remaining MAG+ oligodendrocyteds had lost their cell processes and were reduced to cell ghosts (Fig. 4E). MBP+ oligodendrocytes were only sporadically observed as compared to the controls where many differentiated MBP+ oligodendrocytes were present.

Double Labeling Experiments

Selected infected and non-infected control cultures were used for double labeling studies. In the murine cultures GFAP and MBP were simultaneously visualized, in the canine cultures GFAP and MAG. In the control cultures oligodendrocytes were clearly labeled brown and astrocytes blue; there was no color mixing at all. In the infected double labeled cultures CPE was observed as described above. Cell fusion was clearly present among astrocytes but not among oligodendrocytes. There was no clear evidence for cell fusion between astrocytes and oligodendrocytes.

Discussion

CDV caused a marked CPE in the brain tissue cultures of both species in this study. These changes consisted of cell fusion and cell necrosis with plaque formation in which the culture acquired a reticulated appearance. These changes were similar to those reported by Storts et al. (1968) in the outgrowth zone of CDV-infected cerebellar explant cultures. This process was associated with areas of viral replication as seen by the presence of viral inclusion bodies and CDV antigen. The CPE progressed rapidly and resulted in severe destruction of the culture within 4 days. Both canine and murine astrocytes underwent similar changes. The astrocytes in plaque areas and their vicinity lost their processes, became large plump cells expressing only small amounts of GFAP and fused with adjacent cells. Later on, astrocytes disintegrated, leaving defects in the monolayer. Similar astroglial changes have been observed in CDV-induced demyelinating lesions in vivo, such as multinucleated astrocytes (Summers et al. 1979; Higgins et al. 1982) and marked loss of GFAP+ cells in advanced myelin lesions in acute distemper (Vandevelde et al. 1983). In the earliest detectable lesion in CDV infection in vivo, astrocytic hypertrophy and hyperplasia have been noted (Higgins et al. 1982; Vandevelde et al. 1982). We found no indication of initial progressive astroglial changes in our infected cultures, in contrast to the observed increase in GFAP+ cells in C_6 glioma cell lines after CDV infection (McCormick and Cosby 1981). However, it is questionable whether such observations in tumor cell lines are applicable to the normal astrocyte.

In the canine cultures marked oligodendroglial cell changes were widespread in the culture and not restricted to plaque areas. The lesions consisted of striking irregular distentions of the oligodendroglial perikarya and processes with disruption of the cell membrane leading to cell necrosis. We were not able to document fusion between oligodendrocytes. However, as described before (Zurbriggen et al. 1983), these cells do not form epithelial-like clusters with close cell contact which is typical for astrocytes in culture. Even within large accumulations of oligodendrocytes their perikarya are always clearly separated from each other although they may form contacts at the level of their processes. Viral-induced fusion at this level could only be documented with ultrastructural methods. In the double labeling experiments, where both astrocytes and oligodendrocytes were simultaneously visualized, there was no clear evidence of fusion between the two cell types. Additional experiments, including ultrastructural observations, are needed to confirm this finding. Further evidence of widespread oligodendroglial alteration was provided by the fact that the normal differentiation from MAG+ cells to MBP+ cells (Zurbriggen et al. 1983) did not occur in the infected cultures. Although our study clearly showed severe oligodendroglial changes in CDV-infected canine brain cultures, we are not certain whether this change was the direct result of viral replication within these cells. Only simultaneous immunocytochemical demonstration of viral and specific oligodendroglial antigens may solve this problem. However, while double immunocytochemical labeling for simultaneous demonstration of two antigens in different cell types worked extremely well in our hands, such techniques for visualizing two antigens in one and the same cell are fraught with considerable difficulties. We are presently developing suitable antibodies for this purpose. Ultrastructural methods may also be useful for studying this problem. Whereas astroglial pathology was clearly associated with plaque formation, i.e., areas of initial viral replication, the oligodendroglial changes were distributed throughout the culture soon after infection. This could be due to the fact that oligodendrocytes – being on the surface of the monolayer and separated from each other – may be very accessible to CDV. Another possibility is that the oligodendroglial lesions are secondary to lesions in the other cell types in culture.

This would be more comparable with CDV infection in vivo in which cytolytic infection of oligodendrocytes has not been documented so far (Raine 1976; Summers et al. 1979; Higgins et al. 1982). If the destruction of the canine oligodendrocytes in our study was not due to direct viral cytolysis, then some kind of "bystander effect" could be postulated. The concept of bystander demyelination in vivo has usually been associated with inflammatory lesions, in which enzymes released by activated macrophages in the CNS cause non-specific myelin damage (Wisniewski 1977). Since immunologic reactions were excluded from the in vitro system as used in our study, the oligodendroglial changes could perhaps be explained by release of toxic factors from the infected astrocytes in our cultures.

Of considerable interest was our finding that murine oligodendrocytes were much less affected by CDV infection. We could clearly demonstrate many normal surviving oligodendrocytes even within areas of massive destruction of the murine cultures. Thus, there appears to be a clear difference between the mouse and the dog in effect of CDV infection of oligodendrocytes in vitro. It is possible that this difference my be relevant to the fact that mice, while being susceptible to CDV, do not develop demyelinating lesions after this infection (Lyons et al. 1980; Gilden et al. 1981). The present study has clearly shown that CDV causes considerable oligodendroglial changes in canine brain tissue in vitro. We believe that this finding is of interest with respect to the pathogenesis of CDV-infected demyelination in vivo. The exact nature of this lesion will be studied in subsequent experiments.

Similar studies with other CDV strains will be necessary since there is considerable variation in the biologic effects in vitro as well as in vivo among different CDV strains (Appel and Gillespie 1972; Confer et al. 1975). However, whether such differences in vivo will be reflected in vitro is uncertain since adaptation of CDV to in vitro systems usually results in loss of its virulence and characteristic effects in vivo (Metzler et al. 1980a, b).

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