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Differentiation And Other Factors Influencing The Replication Of Murine Hepatitis Viruses In Cells From The Rat Central Nervous System

Sven Andre Beushausen

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DIFFERENTIATION AND OTHER FACTORS INFLUENCING THE REPLICATION OF MURINE HEPATITIS VIRUSES IN CELLS FROM THE RAT CENTRAL NERVOUS SYSTEM

by

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ABSTRACT

An *in vitro* system employing primary neural cell cultures derived from the cerebral cortices of neonatal rat brains was developed to examine, with greater fidelity, parameters influencing coronavirus (CV) induced demyelination in the rat.

Studies regarding the specificities of the viscerotropic mouse hepatitis virus MHV3 and the sero-related neurotropic JHM virus (JHMV) for neural cell types demonstrated an unambiguous tropism of MHV_3 for astrocytes and JHMV for oligodendrocytes. Relatively small changes in spatial density of oligodendrocytes profoundly influenced JHMV replication. Furthermore, repression of JHMV in oligodendrocytes was shown to be concomitant with differentiation, occurring naturally *in vitro* according to a 'time clock' established *in vivo* or by pretreatment of immature cells with inducers of differentiation that mimic or effect the activation of the adenylate cyclase system.

Examination of the cAMP-dependent protein kinases (PKI, PKII) and respective regulatory subunits (RI, RII) in primary neural cells and rat myoblasts indicated that inhibition of CV replication is correlated with the metabolism of RI. The relationship between CV replication and the adenylate cyclase system prompted an inquiry as to how early cell-virus interactions might be affected by differentiation. Effort were directed at the possibility that protein kinases and/or phosphatases, which participate in cellular regulation during differentiation, were related to CV expression. Although differentiation did not affect virus adsorption and penetration, the

expression of virus-specified RNA and structural proteins was clearly impeded, implying that the block in replication occurs at the stage of uncoating. Evidence is provided suggesting that normal processing of the phosphorylated nucleocapsid (NC) protein is inhibited in differentiated oligodendrocytes.

On the basis of these data it is concluded that in vitro interaction of JHMV with oligodendrocytes accurately reflects the in vivo host control over the tropism and expression of CV. Expression of JHMV is conditional upon the state of differentiation and is interrelated with the adenylate cyclase system. Intracellular accumulation of cAMP, it seems, invokes the modulation of cellular enzymes necessary for divesting the viral genome of the NC subsequently arresting CV replication at uncoating.

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ABBREVIATIONS

2'AMP	adenosine 2'-monophosphate
AMV	avian myeloblastosis virus
ATP	adenosine triphosphate
BME_{10}	basal medium Eagle's with 10% fetal bovine serum
8-Br cAMP	8-bromoadenosine 3':5'-cyclic monophosphate
BSA	bovine serum albumin
^{o}C	degrees celsius
cAMP	adenosine 3':5'-cyclic monophosphate
CB	column buffer
cDNA	complementary deoxyribose nucleic acid
Ci	Curies
cm	centimeter
CNPase	2':3'-cyclic nucleotide-3'-phosphohydrolase
CPE	cytopathic effect
cpm	counts per minute
c.p.s.	a measure of viscosity
CV	coronavirus(es)
dATP	deoxyadenosine triphosphate
dbcAMP	N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate
dbcGMP	N^2,2'-O-dibutyrylguanosine 3':5'-cyclic monophosphate
dCTP	deoxycytidine triphosphate
DEAE	diethylaminoethyl
dGTP	deoxyguanosine triphosphate
DNA	deoxyribose nucleic acid

DTT	Dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) - N,N,N,N,tetraacetic acid
FBS	fetal bovine serum
g	gram(s)
GC	galactocerebroside(s)
GFAP	glial fibrillary acidic protein
G > M-FITC	goat anti-mouse fluorosceneisothiocyanate
G > Ra-RITC	goat anti-rabbit rhodamineisothiocyanate
hr.	hour(s)
IgG	immunoglobulin G
IF	isoelectric focusing
JHMV	mouse hepatitis virus type 4
l	liter
M	molar
MBP	myelin basic protein
µCi	micro Curies
2-ME	2-mercaptoethanol, (β-mercaptoethanol)
MES	(2[N-morpholino] ethanesulfonic acid)
mg	milligram(s)
µg	micrograms(s)
MHV	mouse hepatitis virus
MHV3	mouse hepatitis virus type 3
min.	minute(s)

ml	milliliter(s)
µl	microliter(s)
mM	millimolar
mm	millimeter(s)
µm	micrometer(s)
moles	micromoles
M>MHV	mouse anti-mouse hepatitis virus
MW	molecular weight
N	normal: as in normality of a solution
NC	nucleocapsid
NM	nutrient medium
No.	number
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFU	plaque forming units
PKI	cAMP dependent protein kinase I
PKII	cAMP dependent protein kinase II
PMSF	phenylmethylsulfonyl fluoride
pNPP	para nitrophenyl phosphate
RI	regulatory subunit type I
RII	regulatory subunit type II
R>GC	rabbit anti-galactocerebroside
R>GFAP	rabbit anti-glial fibrillary acidic protein
RIA	radioimmunoadsorbent assay
R>MBP	rabbit anti-myelin basic protein
RNA	ribose nucleic acid

r.p.m.	revolutions per minute
RT	room temperature
SBB	standard binding buffer
SDS	sodium dodecyl sulphate
SSC	standard sodium citrate
ssDNA	single stranded DNA
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
tRNA	transfer RNA
U	units
UV	ultraviolet
V/V	volume per volume
W/V	weight per volume

CHAPTER 1

INTRODUCTION

Naturally occurring virus-induced demyelinative disorders are prevalent among mammals, including man. Although specific agents have been associated with a number of diseases, little is known of the parameters governing the initiation, maintenance and manifestation of the disease process. One approach to investigating such parameters has been through the use of relatively inexpensive animal models.

In vivo models of virus induced demyelinating disease , albeit informative, are very complex with regards to fully understanding the intricacies participating in the disease process. Although virus can often be recovered from infected animals or observed in individual cell types by electron microscopy or viral products detected within identifiable cell types using the techniques of immunofluorescence and in situ hybridization, it is very difficult to envisage or indeed discover the initial host-virus interactions initiating the disease process simply because of the time that has elapsed before clinical symptoms of disease actually are manifested. Certainly one can imagine how the problem becomes even more bewildering if immunologic mechanisms contribute to the progress of disease or in fact maintain the diseased state once the virus has elicited its effect. Many ambiguities concerning the interpretation of such biologic interplay can be circumvented through the use of simplified in vitro neural cell systems representative of the model one wishes to study. Primary neural cell cultures offer the advantage of examining whole or

selected populations of specific cell types comprising the CNS independent of the influence of other systems. The involvement of the immune system, for example, may be studied in conjunction with neural cell culture by the addition of humoral or cellular immunologic components to gain further insight into additional mechanisms contributing to a demyelinative disorder (Bornstein and Appel, 1961; Bornstein, 1963; Raine et al., 1981).

The following presentation deals with an in vitro model of coronavirus induced demyelination in the rat, formerly studied by Sorensen (1982), using cultures of primary rat neural cells. Particular attention has been given to the tropisms of MHV_3 and JHMV for cells of the CNS to account for the disparity in ability to cause disease in vivo. A study has also been directed towards examining the effects of glial differentiation on the replication of JHMV to account for the phenomenon of age-related insusceptibility to virus infection.

CHAPTER 2

2.1 Virus Induced Demyelination

Considerable evidence has been accumulated over the past three decades associating viruses with a number of demyelinating diseases in both man and animals (for comprehensive reviews see: Weiner et al., 1973; Johnson, 1974; ter Meulen and Hall, 1978; Lampert, 1978; Martin and Nathanson, 1979; Lampert and Rodriguez, 1984). Both DNA and RNA viruses have been implicated as etiological agents of certain human CNS diseases. For example, Zu Rhein and Chou (1965) demonstrated the presence of papovavirus particles within the intranuclear inclusions of oligodendrocytes in autopsy material taken from patients with progressive multifocal leucoencephalopathy (PML). Boutelle et al. (1965) and Tellez-Nagel and Harter (1966) first demonstrated virus involvement in the chronic demyelinating disease subacute sclerosing panencephalitis (SSPE) which was later identified as a measles virus (Connolly et al., 1967).

Viruses that infect the CNS are notorious for their ability to become latent or to establish persistent infections. Although a great variety of virus types can produce disease in the CNS, the mechanisms by which latency and persistence are established is only partially understood for a few of the neurotropic DNA and retroviruses belonging to the papova, herpes and lenti-virus groups (for review see Fields, 1986). With the above types of agent, direct integration of viral DNA or provirus intermediates into the host genome provide the mechanism for virus perpetuation. However, conventional mechanisms for

maintaining persistence or latency do not apply to other RNA viruses that do not replicate via a DNA intermediate suggesting that a unique mechanism exists to account for persistent viral infection.

Among the RNA virus types associated with demyelination in animals are retroviruses, including visna virus of sheep (Sigurdsson, 1957) and wild mouse retrovirus causing neurogenic paralysis in mice (Gardner, 1973; Gardner et al., 1976), the picorna agent, Theiler's virus which induces demyelination in mice (Theiler, 1937), mouse hepatitis virus infection of mice and rats by the neurotropic coronavirus JHMV (Cheevers et al., 1949; Bailey et al., 1949), paramyxovirus induced demyelination in dogs by canine distemper virus (Koprowski, 1958; Imagawa et al., 1960), measles virus induced demyelination in hamsters (Wear and Rapp, 1971; Byington and Johnson, 1973), and Semliki Forest virus infection of mice (Chew-Lim, 1975).

Several lines of evidence exist suggesting that one or more of the above animal models may have relevance towards human demyelinating diseases for which there is no known etiology, such as multiple sclerosis (MS). For example, the capability of JHMV to induce a progressive demyelination in the central nervous system of mice and rats, producing foci of demyelination reminiscent of sclera observed in the CNS tissue of patients afflicted with MS (Bailey et al., 1949; Virelizier et al., 1975; Nagashima et al., 1979; Sorensen et al., 1980) has placed the sero-related human strains under consideration as having the potential to trigger this disease. This hypothesis becomes increasingly attractive and certainly merits further investigation in light of a recent report claiming recovery of two coronavirus isolates from autopsy material taken from the brains of persons with MS. The

agents were shown to have extensive serological cross reactivity with the human coronavirus strain OC43 as well as MHV-A59 (Burks et al., 1980) further supporting the notion that agents like CV may be responsible for initiating CNS diseases. Although similar accounts have not been described to corroborate this particular observation, enough circumstantial evidence implicating viruses as the possible etiologic agents of MS exists to warrant further investigation, (Brody et al., 1972; Tanaka et al., 1976).

2.2 Coronaviruses

Members of the coronaviridae are large, pleomorphic, enveloped viruses, approximately 60-220 nm in diameter with a single stranded RNA genome of positive polarity. They are characterized by distinctive, rather widely spaced club-shaped peplomers at the surface of the envelope which are approximately 12-24 nm in length (Robb and Bond, 1969) that have been described as resembling a crown of thorns (Tyrrell, 1968). There are no less than 11 distinct virus types belonging to the group (Siddell et al., 1982) with new isolates bringing this estimate closer to 20. They are of economic and clinical importance because of their pathogenesis in domestic vertebrates as well as man (Siddell et al., 1982).

Members of different virus types can be further subdivided into serotypes. Among them are members of the avian infections bronchitis virus (IBV) (Cowen and Hitchner, 1975), murine hepatitis virus (MHV) (McIntosh, 1974) and human coronavirus (HCV) (McIntosh, 1974; Monto, 1974; Kapikian, 1975). The intraspecies serologic relatedness of the coronaviruses are poorly understood (Tyrrell et al., 1978; Robb and

Bond, 1979; Siddell et al., 1982). However, there is some evidence for antigenic cross reactivity between HCV and MHV (McIntosh, 1974; Kaye et al., 1977).

2.3 Murine Hepatitis Virus

The MHV group consists of a number of serotypes including MHV1, MHV2, MHV3, JHMV, A59, MHV-S, PRI and NuU (Sorensen, 1982). The prototype virus, MHV3, was first described by Dick et al. (1965) and like other members of MHV has an approximate diameter of 100 nm. The genome has an approximate molecular weight (MW.) of 5.4×10^6, is polyadenylated at the 3' end and is by itself infectious (Lai and Stohlman, 1978; Wege et al., 1978). Associated with the virion are three major structural proteins including a large glycoprotein of 180,000 MW., a phosphorylated nucleocapsid protein of approximately 50-60,000 MW., and a smaller envelope glycoprotein of 22-24,000 MW. (Sturman, 1977; Anderson et al., 1979; Bond et al., 1979; Stohlman and Lai, 1979; Wege et al., 1979). Evidence has been furnished suggesting the presence of a protein kinase associated with the virion (Siddell et al., 1981). However, it has not been determined whether this enzyme is encoded by the viral genome or sequestered from the host during assembly.

Following adsorption, infection of cells by MHV is believed to proceed via internalization within endosomes (Krzystyniak and Dupuy, 1984; Mizzen et al., 1985). Events following internalization are unclear. However, treatment of cultures with lysosomotropic agents prior to infection inhibits replication suggesting that continued expression of the virus is dependent on a low pH mediated fusion for

release of the nucleocapsid (NC) into the cytosol, similar to that described for Semliki Forest virus and influenza virus (for review see Mellman et al., 1986).

Replication of virus is independent of host nuclear functions (Brayton et al., 1981) and transcription of the viral genome is preceded by translation in order that the RNA dependent RNA polymerases, necessary for virus replication, can be synthesized (Brayton et al., 1982). There are 6 subgenomic messenger RNAs transcribed from the genome, forming a 'nested set', each with a polyadenylated 3' end (Siddell et al., 1982) and a non-contiguous 72 nucleotide leader sequence ligated to the 5' end (Lai et al., 1983, 1984; Spaan et al., 1983). Recent evidence reported by Makino et al., (1986) suggests that mRNA transcription proceeds via a unique "leader primed transcription" mechanism rather than by splicing of RNA. This was demonstrated by detection of the leader sequences of one virus on the mRNA of another in a mixed MHV coinfection experiment. Ratios of mRNA species, although not transcribed in equimolar amounts, remain relatively constant throughout the course of infection (Siddell et al., 1982).

Translation of virus-specified proteins from the mRNAs proceeds independently within the cytosol of the infected cell (Cheley and Anderson, 1981). The NC protein appears in greatest abundance in infected cell lysates by comparison to other viral polypeptides (Cheley and Anderson, 1981) and massive inclusions containing NC material have been observed in MHV infected L-2 cells by electron microscopy (Massalski et al., 1981). Maturation and assembly of

virions takes place in close proximity to Golgi and endoplasmic reticulum where particles have been observed budding into the cisternae of these organelles (Massalski et al., 1981).

2.4 Coronavirus Induced Demyelination of Rats

The neurotropic variant of MHV, JHMV, was first isolated from two Swiss white mice (Schwentker strain) that were discovered to have flaccid paralysis of the hind legs (Cheevers, et al., 1949). Subsequent investigation regarding the biological properties of the virus demonstrated that recipient mice inoculated intracerebrally developed a hindleg paralysis or an encephalitic disease (Cheevers et al., 1949). Intracerebral inoculation of Cotton rats, Hisaw rats and hamsters also elicited a neurologic disease whereas rabbits and guinea pigs failed to be affected. A study of the pathology of JHMV infected mice led to the discovery that there was a widespread destruction of myelin in the CNS with some focal necrosis of the liver (Bailey et al., 1949). By contrast, in rats the lesions were of a more chronic character, restricted to the CNS, with symptoms appearing at a much later time. (Bailey et al., 1949). Based on these initial findings several laboratories, including our own, have adopted MHV induced demyelination in the rat as a model for studying virus induced demyelinating diseases of the CNS (Nagashima et al., 1979; Sorensen et al., 1980; Hirano et al., 1980).

As in mice (Weiner, 1973) induction of disease, manifestation of symptoms and the type of disease produced in the rat are dependent on a number of parameters including the strain of virus used, the genetic constitution of the host, the age of the host at the time of

inoculation and the route of inoculation (Nagashima et al., Sorensen et al., 1980, 1982; Hirano et al., 1980). Unlike the mouse model where intracerebral inoculation with JHMV, MHV3 or A-59 can initiate varying forms of neurologic disease (Cheevers et al., 1949; Le Provost et al., 1975; Lavi et al., 1984) demyelination in rats was restricted to challenge with JHMV (Nagashima et al., 1980; Sorensen et al., 1980) and in one study with A-59 (Hirano et al., 1980). It is of interest to note that although Hirano et al. (1980) tested JHMV as well as A-59 only the latter elicited a neuropathology consistent with demyelination. Although MHV3 could be recovered from the CNS of 50% of the animals challenged (Hirano et al., 1980) no detectable pathology could be demonstrated in brain tissue examined (Sorensen et al., 1980; Hirano et al., 1980).

Discrimination between the ability of virus types to cause neurologic disease suggested a role for host control over the tropism and expression of MHV within the rat CNS. Indeed, in vitro challenge of the rat Schwannoma cell line, RN-2, (Pfeiffer and Wechsler, 1972) with MHV3 and JHMV somewhat parallelled the in vivo observation whereby the former agent failed to infect the cells and the latter produced a persistent infection (Lucas et al., 1977).

Nagashima et al. (1979) have described the neuropathological findings of three quite distinct CNS diseases observed in rats after intracerebral infection with JHMV. Inoculation of neonates produced a severe panencephalitis with foci of demyelination resulting in the death of almost all animals within 1 to 2 days following the onset of disease. These findings are in agreement with the independent study of Sorensen et al. (1980) who also described a rapidly fatal

encephalitis predominantly involving the gray matter in neonates infected intracerebrally with JHMV. Coronavirus particles of acutely infected animals were observed by electron microscopy in neurons and oligodendrocytes (Nagashima et al., 1979; Sorensen et al., 1980) and antigens were detected in both cell types by immunofluorescence (Nagashima et al., 1979; Sorensen et al., 1984, 1985). Viral RNA was also detected in neurons of acutely infected animals in the regions of the telencephalon and hippocampus by in situ hybridization (Sorensen et al., 1985).

A subacute demyelinating encephalomyelitis was observed approximately three weeks after injection of CHBB/THOM rats inoculated at the time of weaning (Nagashima et al., 1979). The neuropathology of this disease was characterized by demyelination with a striking prediliction for white matter in the brain stem, optic nerve and spinal cord. By contrast to the acute disease, virus was detected by electron microscopy and immunofluorescence in degenerating oligodendroglial cells only. Similar instances of increased involvement of white matter were observed by Sorensen et al., (1980, 1982) in rats inoculated at 10 and 15 days post partum. Histopathology of chronically infected animals revealed pathologic changes in the cerebrospinal axis, pons, cerebellar folia, myelencephalon, spinal cord and the optic nerve. Macrophages were also reported to be numerous in areas of demyelination of chronically infected animals (Sorensen et al., 1980).

A third type of disease described by Nagashima et al. (1979) was a chronic progressive paralysis observed in approximately 25% of inoculated weanling rats that developed 6 to 8 months post-infection

in the absence of a subacute demyelinating encephalitis. The neuropathology was typified by hydrocephalus and myelomacia. However, viral "footprints" could not be detected. The models of Nagashima et al. (1979) and Sorensen et al. (1980) are similarly characterized by a change in neuropathology, predominantly involving the gray matter in the acute disease progressing towards an increased involvement of white matter with demyelinating foci in the chronic diseased states.

An additional, interesting parameter identified by Sorensen et al. (1980, 1982) governing the outcome of JHMV infection was the difference of age-related susceptibility to disease between strains. Outbred Wistar Lewis rats became resistant to infection at approximately 10 days post partum whereas the inbred Wistar Furth strain did not become resistant to infection until the time of weaning. Again, the differences observed in age-related resistance to induction of disease between different rat strains suggested a host control over virus expression, possibly involving differential maturation of the CNS.

The ability of JHMV to persist in the CNS has been adequately demonstrated by the detection of viral RNA using the techniques of in situ hybridization and RNA dot blot hybridization in both asymptomatic and chronically infected animals (Sorensen et al., 1984, 1985). The presence of viral antigen was also detected by immunofluorescence within neurons and glia in areas of the CNS lacking any demonstrable pathology. Furthermore, Sorensen et al., (1982) have shown that exacerbation of inapparent disease can be effected following treatment with cyclophosphamide 28 days after inoculation, implicating the immune system in modulating the disease process. On the basis of

these observations it was concluded that JHMV can persist and possibly even replicate in the CNS for extended periods of time with minimal cell degeneration and death.

In view of the preceding discussion it is apparent that MHV-induced demyelination in the rat is regulated by two very impotant parameters. The first, involves the nature of the discrimination between different strains of MHV to cause a CNS disease within the rat. Since intracerebral inoculation of MHV3 can result in virus replication without overt symptoms of disease (Hirano et al, 1980), there is an implication that this serotype is tropic for cells other than neurons and oligodendrocytes. The second concerns the age-related succeptibility of the rat to infection by JHMV. In order to gain further insight as to the mechanisms operating in the aforementioned parameters, access to a more simplified system, representative of the rat CNS is required.

2.5 Primary Neural Cell Culture

The CNS is comprised of two major cell types, notably the neurons and the neuroglia (for review see Kandel and Schwartz, 1981). Neurons, which vary considerably in shape and the extent of their processes, can be easily distinguished from the neuroglia by their unique morphology. The neuroglia, which provide the housekeeping functions of the CNS, can be further classified into four major types: astrocytes, oligodendrocytes, microglia and ependymal cells. Astrocytes occur in two additional subtypes, fibrous astrocytes and protoplasmic astrocytes. Apart from providing a structural function in maintaining the architecture of the CNS it is believed that

astrocytes also provide a nutritive function to neurons through simultaneous contact between blood capillaries and neuronal membranes. Oligodendrocytes are found predominantly in the white matter of the CNS and serve to insulate the axons of neurons, increasing the velocity of nerve-impulse conduction. The microglial cells are small-ovoid cells that are capable of phagocytosis and hence are often referred to as scavengers of the CNS. Ependymal cells can be found lining the inner surface of the brain.

The in vitro cultivation of neural cells has been exploited as a powerful tool for examining a variety of anatomical, physiological and pathological problems associated with the CNS (Bornstein and Appel, 1961; Bornstein, 1963; Hild, 1966). Sophistication of tissue culture techniques led to the development of aggregating neural cell cultures that provided invaluable information regarding neuronal maturation (Seeds, 1973; Honnegger and Richelson, 1976; Kozak, 1977; Seeds and Haffke, 1973; Trapp et al., 1979, 1982; Lu et al., 1980) glial-cell differentiation (Seeds, 1975; Kozak et al., 1978; Trapp et al., 1979, 1982) and myelination (Sheppard et al., 1978; Trapp et al., 1982).

Relatively simple systems have also been developed for examining neural cell development in dissociated CNS cell cultures. McCarthy and de Vellis (1979, 1980) have described a simple, reproducible method for the culture of mixed glial cells and cultures enriched for either astrocytes or oligodendrocytes from the rat. The advantages of studying a single cell type from a biochemical and pathological point of view are obvious. In addition to the morphologic and ultrastructural criteria that distinguish oligodendrocytes from

astrocytes (Mori and LeBlonde, 1969, 1970; McCarthy and de Vellis, 1980) biochemical and antigenic markers can be used to identify and monitor the differentiation of these cell types in vitro.

Astrocytes can be cytochemically identified by an abundance of glial fibrillary acidic protein (GFAP) first described by Eng et al. (1970), later shown to be localized to the intermediate filaments within the cell by immunofluorescence (Bignami et al., 1972). GFAP is present only in mature astrocytes, replacing vimentin as the intermediate filament protein in the maturing astrocyte at the time of myelination (Dahl, 1981). Increased production and reorganization of GFAP has been observed in astrocytes undergoing natural differentiation in vitro (Trimmer et al., 1982) or in cells induced to differentiate with dibutyryl cyclicAMP (dbcAMP) (Goldman and Chiu, 1984). The presence of GFAP is not confined to astrocytes as it has also been detected in immature oligodendroglia, enteric glia and Schwann cells (Choi and Kim, 1984, 1985; Jessen et al., 1984; Ogawa et al., 1985). This anomaly can be explained for oligodendroglia since oligodendrocytes develop from O-2A bipotential progenitor cells that become type 2 astrocytes, identified by the presence of GFAP, prior to differentiating and acquiring antigens typical of oligodendrocytes (Raff et al., 1983a, 1983b; De Los Monteros et al., 1985; Raff et al., 1985; French-Constant and Raff, 1986; Temple and Raff, 1986).

Oligodendroglia can be identified by a number of immunocytochemical and biochemical criteria in vitro including the membrane glycolipid galactocerebroside (Raff et al., 1978), myelin basic protein (Bhat et al., 1981; Barbarese and Pfeiffer, 1981; Pfeiffer et al., 1981) and the myelin related enzyme 2':3'-cyclic

nucleotide 3-phosphohydrolase (CNPase) (McCarthy and deVellis, 1980; Bhat et al., 1981; Pfeiffer et al., 1981; McMorris, 1983; Bansal and Pfeiffer, 1985). Neural cells, including oligodendrocytes, differentiate in vitro according to a time clock established in vivo (Abney et al., 1981). Consequently, a considerable amount of work has been directed at studying the development of oligodendrocytes in vitro (Raff et al., 1983; De Los Monteros et al., 1985; Raff et al., 1985; Ffrench-Constant and Raff, 1986; Temple and Raff, 1986) and the regulation of myelinogenic parameters (Bhat et al., 1981; Barbarese and Pfeiffer, 1981; Pfeiffer et al., 1981; Cammer et al., 1982; Ranscht et al., 1982; McMorris, 1973; Singh and Pfeiffer, 1985; Bansal and Pfeiffer, 1985; Wernicke and Volpe, 1985). Moreover, cultured neonatal rat oligodendrocytes are capable of elaborating myelin in the presence or absence of neurons, further testament to their capacity for differentiation in vitro (Wood et al., 1980; Bradel and Prince, 1983; Wood and Williams, 1984; Nishimura et al., 1985). Oligodendrocytes induced to differentiate with dbc AMP show demonstrable increases in the specific activities of lactate dehydrogenase, glycerol-3-phosphate dehydrogenase, and CNPase, as well as an increase in the expression of the differentiation antigens myelin basic protein and galactocerebroside (McCarthy and de Vellis, 1980; McMorris, 1983; Wernicke and Volpe, 1985). It is of interest to note that Wernicke and Volpe (1985) observed a profound retardation of the amounts of temporally induced enzymes, indicative of differentiation, in oligodendrocytes plated at high density. This

phenomenon was not observed to occur in astrocytes suggesting that the degree of differentiation of oligodendrocytes is closely regulated by spatial density of the cells.

Clearly, the expression and nuances of differentiation unique to both astrocytes and oligodendrocytes *in vitro* make them suitable models for studying virus-cell interactions relevant to CV induced demyelination in the rat CNS.

2.6 Objectives of the Study

The purpose of this study was to examine the early host-virus interactions of MHV-induced demyelinating disease of the rat *in vitro* and to extrapolate the findings back to the *in vivo* model, formerly described by Sorensen (1982). For this purpose, cultures of primary neural cells from the rat CNS were established, consisting primarily of mixed glial cells or cultures enriched for either astrocytes or oligodendrocytes, and challenged with MHV3 or JHMV. The neurotropic JHMV was studied in greater detail because of its ability to elicit a CNS disease in the rat and as such, parameters influencing the disease process *in vivo* were addressed and scrutinized *in vitro*. Emphasis was placed on the phenomenon of age-related insuceptibility to JHMV infection *in vivo* by asking questions of how differentiation of oligodendrocytes *in vitro* affects JHMV replication. Once it was established that differentiation repressed virus replication, the focus of the work was directed towards investigation of metabolic machinery related to differentiation participating in JHMV inhibition.

CHAPTER 3

MATERIALS AND METHODS

3.1 Continuous Cell Lines

L-2 murine fibroblasts (Rothfels et al., 1959), LMTK$^-$ cells (Merchant et al., 1962) derived from the L929 line (Sanford et al., 1949), L6 rat myoblasts (Yaffe, 1968), and the spontaneously transformed myoblast line JRU5 (Seth et al., 1983) were routinely propagated as monolayers in Eagle's minimum essential medium (Gibco, Grand Island, N.Y.) supplemented with 5% fetal bovine serum, (FBS) (Whittaker Bioproducts, Walkersvillem MD) penicillin (100 u/ml), and streptomycin (100 µg/ml), termed nutrient medium (NM), at 37^{o}C in an humidified atmosphere containing 5% CO_2.

3.2 Primary Neural Cell Cultures

Primary rat neural cell cultures of oligodendrocytes and astrocytes were prepared using a simplification of the method patterned after that of McCarthy and de Vellis (1978, 1980). Following removal of the meninges, the cerebral hemispheres from 1 to 2 day old Wistar Furth rat pups were placed in a 100 mm dish containing ice-cold basal medium eagle (BME_{10}, Gibco) supplemented with 10% FBS, 0.6% dextrose and 10 µg/ml gentamycin (Gibco). The hemispheres were rinsed 3 times with ice-cold BME_{10} and then triturated through a 10 ml pipette until a uniform suspension, free from large particulates, was obtained. The resulting slurry was

passed through a nylon mesh of 130 μm pore diameter (Nitex 130, Tetco Inc., Elmsford, N.Y.) and the filtrate, containing dispersed cells, centrifuged at 40 x g for 5 min. at 4°C. The supernatant was removed and the cell pellet resuspended in 10 ml of BME_{10}. The suspension was aliquoted such that cells derived from the cerebral hemispheres of 5-6 animals were seeded onto a 180 cm^2 tissue culture flask. The flasks were incubated in an humidified atmosphere containing 5% $C\theta_2$ at 37°C for at least four days before changing the medium, to allow the cells to settle onto the substratum. The cells were subsequently refed with fresh BME_{10} every second day after the initial four day incubation.

To obtain cultures enriched in oligodendrocytes or astrocytes, the mixed cultures were manipulated on the eighth day after explantation as described by McMorris (1983). Mixed cultures were replenished with 30 ml of fresh BME_{10} and allowed to equilibrate at 37°C for a minimum period of 2 hours (hr.). Following equilibration, the flasks were vigorously shaken by hand and allowed to stand for a few minutes. The less tightly adherent cells, which were predominantly of the oligodendrocytic type, became freed into the medium. The freed cells were then filtered through a Nitex 130 mesh and plated onto 80 cm^2 flasks at a density of 2-3 x 10^7 cells/flask. To further enrich for oligodendrocytes, the shaken cells were allowed to adhere to the substratum for 24 hr. They were shaken manually again and replated at a final density of 2-3 x 10^5 cells/cm^2. Cultures enriched for astrocytes were prepared from the remaining adherent cells following the initial shaking of the mixed cultures. The flasks, reconstituted with fresh BME_{10}, were incubated at 37°C

until the cells had formed monolayers. Once formed, the monolayers were rinsed 3 times with warmed citrate saline (pH 7.2) and shaken by hand to remove any contaminating oligodendrocytes. The remaining cells, predominantly of the astrocytic type, were suspended in 20 ml of BME_{10} by vigorous shaking after treatment with 0.25% trypsin (Gibco) in citrate saline for 1-2 min at room temperature RT. The cells were then enumerated and plated at the desired working density.

3.3 Viruses and Preparation of Stocks

Mouse hepatitis virus strains MHV3 and [redacted], originally obtained from the American Type Culture Collection (Rockville, MD), (Lucas et al., 1977) were grown on monolayers of L-2 cells. Virus was adsorbed onto L-2 cells at RT with periodic rocking for 60 min. then incubated at 37°C. Once the infection had progressed to the stage when 50% of the syncytia had lifted off, the remainder of the fused monolayer was scraped off with a rubber policeman and the suspension was taken up into a syringe and forced through a No. 30 guage needle, to cause disruption. The lysates produced were centrifuted at 40 x g for 5 min. at 4°C and the supernatant filtered through a millipore Millex-GS, 0.22 μm filter unit to remove larger debris. The filtrate, dispensed in 0.5 ml aliquots, was stored at -70°C or used when fresh.

The Indiana strain of vesicular stomatitis virus (VSV) was grown on monlayers of L-2 cells for approximately 24 hr. and the virus containing supernatant fluid was used as with CV.

3.4 Methods for Inoculation and Assaying Infectivity

Primary neural cell cultures were inoculated with virus stocks, suspended in BME_{10}, at 0.1 ml/cm^2. Adsorption was allowed to proceed for 60 min. at the desired temperature with periodic rocking. The multiplicity of infection (m.o.i.) was varied, depending on the experiment, as indicated in the figures and tables presented in Results. Three to four hours after adsorption, surface and extracellular inoculum was neutralized using highly active antiviral antisera at a dilution of 1:124 in BME_{10} for 30 min. at 37°C. Following neutralization the cells were washed, then reconstituted with BME_{10}, and incubated at the desired temperature.

L6 rat myoblasts and the spontaneously transformed JRU_5 myoblasts were routinely infected in 6 well multiwell dishes using 1 ml of inoculum. Adsorption, neutralization of extracellular virus, and incubation of cells was carried out as described above with the exception that the cells were maintained in NM.

CV concentrations were determined as plaque-forming units (PFU) on L-2 cell monolayers grown in 6 well multiwell dishes. Ten fold serial dilutions of the sample to be tested were made in NM and 0.25 ml aliquots of each adsorbed onto L-2 cells for 60 min. at RT with constant rocking. Each well was overlayed with 3 ml NM containing 5 mg/ml carboxymethyl cellulose (4000 c.p.s.) and incubated at 37°C for 18-24 hr. Fixation of cells was achieved by adding 1 ml of 37% formaldehyde directly to each well. After approximately 10 min., the NM-formaldehyde solution was aspirated from the wells and the cells stained with a solution of 0.1% crystal violet for 10 min. at RT. The

excess stain was removed by thorough washing in running water and the plates were air-dried. Wells containing between 20 to 200 plaques, typical of CV syncitia, were enumerated. VSV production was monitored in a similar fashion.

3.5 Preparation of Antisera to Coronaviruses

CV antisera to MHV3 and JHMV (M>MHV) were raised in Swiss mice. Neonates were tolarized against once frozen and thawed L-2 cells by intraperitoneal injections with approximately 1×10^7 cells per animal in 0.05 ml phosphate buffered saline (PBS), containing 8.775 g/l NaCl, 6.125 g/l KH_2PO_4 and 1.025 g/l NaOH (pH 6.8), at 24 and 48 hr. postpartum. Two weeks later the pups were reinjected with the same material to maintain tolerance. Four weeks postpartum the mice were injected intraperitoneally four times, at weekly intervals, with concentrated UV inactivated virus, which had an initial titre of approximately 5×10^8 PFU/ml. One week after the final injection, the blood drawn from batches of animals was pooled, the serum separated, diluted 1:4 in PBS, and stored at $-70^{\circ}C$. Specificity of the antisera was tested by indirect immunofluoresce on CV infected L-2 cells. Antisera that demonstrated specificity for cellular antigens were adsorbed against acetone precipitated L-cells to remove any of the non-virus-specific antibodies.

3.6 Preparation of Antisera to Galactocerebrosides

Antisera to galactocerebrosides were (GC) prepared in 2 adult, New Zealand albino rabbits (R>GC) according to the method of Raine et al. (1976). The antigen mixture for the primary inoculation contained 5 mg of mixed GC from bovine brain (Sigma, St. Louis, MO), 0.5 ml of micelles made up of equal parts of lecithin and bovine serum albumin (BSA), and 0.5 ml of complete Freund's adjuvant. Three additional boosters were injected at biweekly intervals and contained 1 mg of GC emulsified with the lecithin-BSA micelles in incomplete Freund's adjuvant. All injections were made subcutaneously in the rear flank and neck region and sera were collected one week after the final inoculation.

3.7 Purification of Antibodies to Galactocerebrosides

Specific antibodies to GC were recovered from the crude sera by sequential passage through protein A-sepharose and BSA-sepharose columns, respectively, to recover the IgG fraction and eliminate the anti-BSA antibodies. Protein A and BSA were coupled to cyanogen bromide activated sepharose 4B (Pharmacia, Uppsala, Sweden) at an approximate concentration of 5 mg/ml. The protein-coupled sepharose slurries were packed into minicolumns (Bio Rad, Richmond, CA) to a volume of 1.5 ml and equilibrated with 100 mM phosphate buffer made to pH 8.0 by mixing 100 mM Na_2HPO_4 with 100 mM NaH_2PO_4.

Initial enrichment for IgG antibodies was carried out by loading approximately 6 ml of crude anti-GC antiserum onto the protein A-sepharose column and eluting the bound IgG from the column with 100 mM

glycine buffer (pH 3.0). The eluent was collected in 0.5 ml fractions into tubes containing 0.5 ml of 100 mM Tris-HCl (pH 8.0). Fractions were analyzed for protein content in a spectrophotometer at 280 nm and fractions with the highest optical densities were pooled and dialyzed by amicon filtration using a PM10 membrane against 100 mM phosphate buffer. The final volume of the retentate, containing the IgG fraction of the antiserum, was 3 ml.

Further enrichment for anti-GC antibodies was achieved through the elimination of anti-BSA antibodies by affinity chromatography on a BSA-sepharose column. The IgG fraction obtained from the protein A-sepharose column was passed over a BSA-sepharose column and the anti-GC containing eluent collected and concentrated by amicon filtration. Anti-BSA antibodies were eluted from the column with 100 mM glycine buffer and discarded. Columns were requilibrated with 100 mM phosphate buffer and stored at 4°C in 100 mM phosphate buffer containing 0.1% sodium azide.

3.8 Avidity of Anti-Galactocerebroside Antibodies

The avidity of R>GC antibodies was assessed using the radioimmunoassay (RIA) of Holmgren et al. (1980) for detecting gangliosides adsorbed onto plastic wells, as modified by Raine et al. (1981) for the detection of GC. Antigen was prepared by solubilizing 5 mg of mixed GC and 17.5 mg each of lecithin and cholesterol in 1 ml of 95% ethanol, at 56°C for 5 min. To obtain a uniform dispersion of GC-lecithin-cholesterol micelles (Marcus and Schwarting, 1976) 4 volumes of PBS was added and the resulting suspension was sonicated

for several minutes. The dispersed micelles were diluted 1:99 in PBS and 100 µl applied to each of several wells of a 96 well, soft plastic, microtitre plate. The lipid micelles were adsorbed to the plastic by standing overnight at RT. The wells were washed with PBS containing 0.05% Tween 20 (PBS-Tween) several times followed by incubation for 2 hr. at RT with buffer containing PBS-Tween and 1% BSA to block nonspecific immunoreactive sites. The wells were washed 3-4 times with PBS-Tween followed by inundation with 50 µl of various antisera applied at differing dilutions, in PBS, in triplicate. Following incubation for 2 hr. at RT, the wells were washed 5-6 times with PBS-Tween, then incubated in the presence of 50 µl ^{125}I-protein-A (10 µCi/ug, New England Nuclear) diluted in PBS to approximately 600 cpm/µl. After incubation for 2 hr. at room temperature the wells were washed several times with PBS-Tween then air dried. Each well was cut from the dish and the amount of ^{125}I protein A binding to each well quantitated in a gamma counter.

3.9 Immunelabelling of Neural Cells

Rabbit antiserum to guinea pig myelin basic protein (Ra > MBP) was kindly provided by Zobeeda Hossein. Rabbit anti-human glial fibrillary acidic protein (Ra > GFAP) (Dahl et al., 1976) was a gift from Dr. Doris Dahl.

Cells for examination by immunofluorescence were grown on 12 mm coverslips (Chance, Propper, Smethwick, Worley, England, No. 1) in 24 well trays. Cultures to be processed for labelling of surface antigens were fixed in 1% formaldehyde in PBS for 10 min. at RT

whereas cells to be stained for cytoplasmic antigens were fixed by immersion in acetone for 2 min. at RT, as described by Manthorpe et al. (1979). Manipulations prior to and immediately following fixation were identical for the labelling of surface and intracellular antigens. Prior to fixation the cells were rinsed once with warmed BME_{10} followed by 2-3 rinses in warmed PBS. Following fixation, the coverslips, complete with fixed cells, were washed extensively by immersion into 4 baths of PBS for approximately 5 min. in each bath. Coverslips ready for labelling were individually placed in each well of a 6 well dish, inundated with 50 μl of the primary antibody, (or antibodies in the case of double labelling) and incubated for 30 min. at RT. Primary antibodies, unbound to their respective antigens, were washed away with 6 x 5 ml washes of PBS. The fluorescence labelled antibodies, goat anti-rabbit conjugated to rhodamine (G>Ra-RITC) and goat anti-mouse conjugated to fluorescene (G>M-FITC) (Miles Biochemicals, Elkart, Ind.) were diluted in PBS and applied individually or together as required immediately following the washing and incubated for 30 min. at RT. Unbound antibodies were washed away with 6 x 5 ml washes of PBS. Coverslips were mounted on glass slides with an aqueous solution containing 90% glycerol, to maximize fluorescence, and examined and photographed under UV optics using a wild-Leitz, Dialux 20 microscope.

The antisera were used in the following final dilutions: R>GC, 1:1; R>MBP, 1:9; M>MHV, 1:4, R>GFAP, 1:39, G>Ra-RITC, 1:19; G>M-FITC, 1:19.

3.10 Immune Lysis of Infected Cells

Primary oligodendrocyte or astrocyte cultures and LMTK$^-$ cells were infected, monitored for development of cytopathic effect (CPE) then treated with Ra>GC antibody plus complement to cause lysis. The anti-GC antibodies diluted 1:8 in NM were applied at 4°C to infected cultures for 60 min. After rinsing away the unabsorbed antibodies with 3 washes of cold BME_{10}, the cultures were overlayed with rabbit Lo Tox complement (Cedarlane, Hornby, Ontario, Canada), diluted 1:14 in NM and placed in an humidified CO_2 incubator for 30 min. The unfixed complement was removed with 3 rinses of warm BME_{10}. The cultures were reconstituted with BME_{10} and incubated at 37°C for 24 hr to determine virus yields in terms of PFU/ml in the medium.

3.11 Preparation of Neural Cell Cultures for Electron Microscopy

The procedure for embedding neural cell cultures *in situ* for examination by electron microscopy was similar to that described by Brinkley *et al.* (1967). Monolayers of mixed neural cell cultures and cultures enriched in oligodendrocytes or astrocytes were rinsed twice with warmed BME_{10} prior to fixation with 4% glutaraldehyde in BME_{10} for 1 hr. at RT. Following fixation, the monolayers were washed twice with PBS then post-fixed in 1% OsO_4 for 30 min. at RT. Residual OsO_4 was removed with 3 washes of deionized H_2O and the monolayers were prestained for 30 min. with 2% aqueous uranyl acetate. Prior to dehydration, the monolayers were rinsed twice with deionized water then subjected to a dehydration series consisting of two 10 min. changes in 35%, 50%, 75%, 90% and absolute ethanol. The final

dehydration steps involved filling the flasks or plates with absolute alcohol and incubating overnight at 4°C followed by a 10 min. exchange of absolute ethanol the next morning. Infiltration of the cells with Epon was initiated using 75% Epon in absolute alcohol, 2 changes each 30 min. in duration followed by a third change left overnight at RT. The cells were then infiltrated with pure Epon following the same regimen. Polymerization was achieved by placing the cells, covered with a thin film of Epon, in an oven and incubating overnight at 60°C. It was imperative that the entire top of the flasks were removed or well ventilated to facilitate evaporation of the residual alcohol from the Epon. After polymerization, the monolayer was peeled from the flask, examined under a light microscope, and areas to be sectioned for electron microscopy were cut out and placed bottom side up in a small dish. The sections were overlayed with fresh epon and polymerized at 60°C to make an Epon sandwich. The sandwiches were cut, trimmed then sectioned on a Porter-Blum microtome. Sections were examined in a Philips 300 electron microscope.

3.12 Treatment of Cell Cultures with Inducers of Differentiation

Primary astrocytes and oligodendrocytes as well as cultures of L-2 fibroblasts and L6 myoblasts were treated with N^6, 2'-O-dibutyryl-adenosine 3':5'-cyclic monophosphate (dbcAMP), 8-bromoadenosine-3':5'-cyclic monophosphate (8-Br cAMP), N^2, 2'-O-dibutyrylguanosine 3':5'-cyclic monophosphate (dbcGMP) and papaverine, purchased from Sigma, St. Louis, MO, or with isoproterenol (Isuprel, Winthrop Labs, Aurora, Ontario, Canada), or forskolin, supplied by

Calbiochem, Behring Diagnostics, San Diego, CA. The metabolites were diluted in BME_{10} and applied at varying concentrations and for various intervals, depending on the experiment.

Time-course studies on the interrelationship between concentration of the regulatory subunits RI and RII of the cAMP dependent protein kinases, PKI and PKII and dbcAMP treatment were carried out as follows: astrocytes in 100 mm culture dishes were seeded in BME_{10} at a density of 5×10^6 cells/plate and oligodendrocytes, in 25 cm^2 flasks at a density of $3\text{-}5 \times 10^6$ cells/flask. The medium, plus dbcAMP, was changed daily until the time of harvesting. Duration of exposure to dbcAMP was varied by commencing dbcAMP treatment of duplicate cultures on succeeding days, whereby incubation in the presence of dbcAMP ranged from 5 to 0 days. By staggering the day on which treatment was initiated it was possible to harvest all the cultures simultaneously. Consequently, all cultures employed in these studies were of the same age.

3.13 Preparation of Cell Extracts

Neural cells to be assayed for 2':3'-cyclic nucleotide 3'-phosphohydrolase were grown in 60 mm dishes. At the time of harvesting, cells were rinsed 3 times with 0.15 N NaCl, scraped from the substratum with a rubber policeman and centrifuged at 150 x g for 2 min. at 4^oC. the cell pellets were resuspended in 500 μl of deionized water containing 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). Extracts were prepared by mixing 300 μl of the suspension with 2 volumes of 1% sodium deoxycholate in H_2O. The samples were

left on ice for 10 min. prior to homogenizing with 20 strokes in a Potter-Elvehjem homogenizer. The extracts were aliquoted and stored at -70°C. The 200 ul aliquots remaining from the original suspensions were assayed for protein content by the method of Lowry et al. (1951), using BSA as the standard.

To prepare cytosol material for photoaffinity labelling of the PK regulatory subunits the primary neural cells and continuous lines were propagated as above. Briefly, 3×10^6 oligodendrocytes grown in 25 cm^2 flasks or 5×10^6 astrocytes or L6 myblasts propogated in 100 mm dishes were rinsed twice with ice-cold PBS. Oligodendrocytes were harvested by manual shaking into 5 ml of PBS. Astrocytes and myoblasts were scraped off with a rubber policeman and suspended in 5 ml of ice-cold PBS. The cells were collected into pellets by centrifugation at 150 x g for 10 min. at 4°C, then were resuspended in 600 ul of ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 5 µg/ml PMSF and 2 µg/ml leupeptin followed by disruption with 40 strokes in a Potter-Elvehjem homogenizer. The homogenates were clarified by centrifugation at 100,000 x g for 60 min., 4°C and the supernatant was designated as the cytosol fraction.

Cytosol material for DEAE-cellulose column chromatography was prepared as previously described by Rogers et al. (1985). Briefly, the pellets of $2\text{-}3 \times 10^8$ cells were resuspended in 3-4 ml of buffer, containing 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5mM EGTA, 1 mM PMSF, 2 µg/ml leupeptin and 0.1% B-mercaptoethanol (2-ME) and disrupted after 20-30 strokes in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 40,000 x g for 60 min. The

supernatant fractions obtained were dialyzed overnight against buffer A, containing 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) (pH ~6.7), 1 mM EGTA, 2 μg/ml leupeptin, 1 mM PMSF and 0.1% 2-ME.

Oligodendrocyte and astrocyte cultures, seeded at a density of 2-3 x 10^7 cells/75 cm^2 flask, were used to prepare supernatant and particulate fractions for assaying phosphatase activities. Following incubation in BME_{10} or BME_{10} plus differentiation inducers the cells were harvested as described for photoaffinity labelling. The supernatant fractions derived from 100,000 x g sedimentation were stored at -70°C. The remaining particulate fractions were rinsed gently 3-4 times with 10 mM Tris-HCl (pH 7.4) buffer containing 1 mM EGTA, 5 μg/ml PMSF, and 2 μg/ml leupeptin, then resuspended in 500 μl of the same buffer with added 1% triton X-100 (Tris-Triton), and dispersed to homogeneity by 40 strokes in a Potter-Elvehjem homogenizer. The resulting suspensions of cell fragments were also stored at -70°C.

The protein content in all fractions was determined according to the method of Lowry et al. (1951), using BSA as the standard.

3.14 Assay of 2':3'-Cyclic Nucleotide 3'-Phosphohydrolase

The procedures used for determining levels of the enzyme 2':3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) in primary neural cell cultures were those developed by Prohaska et al. (1973) as modified and described by McMorris (1983). The reaction mixtures for CNPase activity containing 100 μl of cell extract, adjusted to 20 μg protein with H_2O, and 100 μl of 50 mM Tris-HCl (pH 6.2) containing 15 mM

2':3'cAMP were incubated for 10 min. at 30°C. CNPase activity was terminated by placing the reaction mixtures in boiling water for 1 min. Phosphate was liberated from the 2'AMP reaction product by adding to the reaction mixtures 100 µl of 100 mM glycine buffer (pH 10.2) containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 2.5 U of calf intestine alkaline phosphatase (Boehringer-Mannheim, 2500 u/mg) and incubating for 30 min. at 30°C. The inorganic phosphate liberated was extracted by adding 1.2 ml of isobutanol:benzene, 1:1, followed by the addition of 1.2 ml 1.5% ammonium molybdate (Aldrich) in 0.5 N H_2SO_4. The solutions were vortexed for 20 sec. then centrifuged at 650 x g for 5 min. and the yellow upper layer read in a spectrophotometer at 410 nm against the isobutanol:benzene blank. Standard phosphate carried through this procedure generates a linear relation of 0.75 umoles/absorbance unit.

One unit of enzyme activity is that amount which produces 1 umole of 2'-AMP from 2':3'-cAMP/min. under the conditions described.

3.15 DEAE-Cellulose Column Chromatography

DEAE-cellulose columns (1.5 x 10 cm), 3 to 5 ml bed volume, were equilibrated with approximately 5 volumes of Buffer A prior to loading of samples in accord with the method of Rogers et al. (1985). Separation of PKI and PKII was accomplished by loading 2 to 5 µg of 40,000 x g supernatant fractions onto the columns followed by washing with 5 volumes of Buffer A and subsequent elution of the enzymes with

a 100 ml linear gradient of (0-0.4M) NaCl in Buffer A. Single ml fractions were collected and assayed for cAMP-dependent protein-kinase and [^{3}H] cAMP binding activities.

3.16 Measurement of cAMP Dependent Protein Kinase and [$^{-3}$H] cAMP Binding Activities

Protein kinase activity was measured by the method of Roskoski (1983) with some modifications. The protein kinase reaction mixture contained 20 mM Tris-HCl, pH 7.5, 125 nM [γ^{32}p] ATP (3000 Ci/m mol, NEN) diluted to 100 cpm/pmol, 5 mM magnesium acetate, 32.5 mM Kemptide (Sigma), 0.25 mg/ml BSA, 10 mM NaF, H_2O and enzyme to give a final volume of 70 µl. Samples were incubated for 10 min. at 30°C in the presence and absence of 3 mM cAMP. Following incubation, 50 µl aliquots were withdrawn, spotted onto 2 x 2 cm phospho-cellulose paper strips (Whatman P81) and immersed in 75 mM phosphoric acid. The strips were washed in phosphoric acid 4 times (5 min. each) with stirring followed by one rinse in 95% ethanol and dried under a heat lamp. The radioactivity was measured in a liquid scintillation counter. One unit is defined as that amount of enzyme catalysing the transfer of 1 pmol of phosphate from ATP to Kemptide/min.

[^{3}H] cAMP binding activity to the regulatory subunits of PKI and PKII was determined using the method of Gilman (1971) with modifications employed exactly as described by Rogers et al. (1985).

3.17 Photoaffinity Labelling of RI and RII

Covalent linking between RI and RII and the photosensitive cAMP analogue, 8-azido-[^{32}P]cAMP. (45 Ci/mM, ICN, Irvine, CA) was carried out according to the method of Walter et al. (1979). The reaction mixture contained 50 mM MES (pH 6.2), 10 mM $MgCl_2$, 1 mM EGTA, 2 µg/ml leupeptin, 50 µg/ml PMSF, 25 nM 8-azido-[^{32}P] cAMP and 5 µg cytosol protein in 100 µl. The reaction was initiated at RT for 60 min. in the dark. Then the samples, placed on ice, were irradiated for 10 min. by means of a UVS-11 hand lamp, at a distance of 10 cm. Following irradiation, Laemmli sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970) was added to each reaction mixture and samples were prepared for electrophoresis. To demonstrate the specificity of the coupling reaction controls were also incubated in the presence of a 50:1 mixture of cold cAMP:8-azido-[^{32}P] cAMP.

3.18 Analysis by SDS-Polyacrylamide Gel Electrophoresis

Samples were analyzed on 10% polyacrylamide gels with 0.1% SDS according to the method of Laemmli (1970). Gels were dried under vacuum and bands visualized by autoradiograms using Kodak XAR-5 X-ray film. Quantitative estimates were made from the autoradiograms by means of a LKB Ultrascan SL laser densitometric scanner. The relative amounts of label were compared according to the area occupied by individual peaks in the scan.

3.19 Adsorption and Penetration of Virus.

For estimating adsorption, triplicate cell cultures were seeded on 35 mm dishes in BME_{10} and allowed to equilibrate overnight at 37°C prior to manipulation. Ce density at the time of plating varied depending on the cell type sec, as evident in Results. To determine adsorption efficiency, cultures were placed on ice, rinsed twice with ice-cold BME_{10} and overlayed with 1 ml of cold BME_{10} containing 2 x 10^7 PFU JHMV to deliver at least 10 PFU/cell. Adsorption, using periodic rocking, was for 60 min. at 4°C. Following adsorption, the unattached virus was removed by rinsing three times with PBS-Tween. The cells were then scraped from the substratum with a rubber policeman and forced through a 30 gauge needle, causing cellular disruption and dispersal of adsorbed virus on the cell fragments. Immediately after disruption the virus was titrated on L-2 monolayers.

In studies of penetration, triplicate oligodendrocyte cultures were plated on 35 mm dishes at an initial density of 1.5 x 10^6 cells/dish and overlayed with BME_{10} or BME_{10} plus 1 mM dbcAMP. Following a 48 hr. incubation at 37°C the cultures were rinsed twice with ice-cold BME_{10} and overlayed with 1 ml of warmed BME_{10} containing sufficient JHMV to deliver at least 10 PFU/cell. Virus penetration was ascertained by rinsing cell-virus complexes 3 times with PBS-Tween, then adding 2 ml of proteinase-K (Boehringer-Mannheim) in PBS (0.5 mg/ml) and incubating at 4°C for 45 min to inactivate extracellular virus. After incubation in the cold, 1 ml of PBS, containing 1% BSA and 2 mM PMSF, (PBS-PMSF), was added to inhibit protease activity and the entire suspension transferred into a conical

centrifuge tube. After centrifugation at 650 x g for 2 min. at 4°C the cell pellets were resuspended in PBS-PMSF and recentrifuged twice to remove any free virus, then were pelleted as above, resuspended in 1 ml of PBS containing 0.2% BSA and forced through a 30 gauge needle. The cell-associated virus present with the cell fragments was titrated on L-2 monolayers.

3.20 Synthesis of [^{35}S]-Labelled DNA Complementary to JHMV RNA.

Complementary DNA (cDNA) was synthesized according to the method of Coulter-Mackie et al. (1980), using JHMV RNA (kindly supplied by Dr. O. Sorensen) as the template (Sorensen et al., 1986). The reaction mixture consisted of 5 µl of a salt- nucleotide mix containing 0.25 M Tris HCl (pH 8.0), 40 mM $MgCl_2$, 1.2 mM each of dATP, dGTP and dTTP, 0.25 M KCl and 10 mM dithiothreitol (DTT), mixed with 5 µl of calf thymus primers, 4 µl of actinomycin D (1mg/ml), 2 µl of AMV reverse transcriptase (Life Sciences), 4 µl deionized H_2O, 10 µl [^{35}S] dCTP (1258 Ci/mmole, New England Nuclear) and 5 µl of JHMV template RNA, which had been boiled for 2 min. and rapidly quenched prior to addition to the reaction mixture. After incubating the mixture at 37°C for 45 min., 0.5 µl of 3 mM dCTP was added and the incubation resumed for another 15 min. Alkaline hydrolysis of RNA was achieved by adding 13 µl of 2N NaOH to the mixture and incubating for a further 2 hr. at 37°C. The mixture was subsequently neutralized by the addition of 13 µl 2N HCl and the DNA products were chromatographed on a 45 x 1 cm G-75 sephadex column, which had previously been equilibrated with column buffer (CB) containing 0.30 M NaCl, 0.02 M

Tris HCl (pH 7.4), 3 mM EDTA, 0.1% SDS, and 10 μg/ml yeast RNA. Approximately thirty, 1 ml fractions were collected and aliquots from each tested for radioactivity. Fractions with the highest cpm were pooled, mixed with 10 μl of 10 mg/ml yeast RNA and the nucleic acid in them precipitated overnight at -20°C by the addition of 2 volumes of 95% ethanol.

The precipitated labelled DNA was sedimented at 16,000 x g for 60 min. at -10°C. Liquid was removed by draining the tube and the pellet resuspended in 0.5 ml of 0.9 mg/ml L-2 cell DNA, which was added to remove by annealing any contaminating cell-specific probe. This mixture was immersed in a boiling water bath for 3 min., then rapidly quenched in ice water. Hybridization was allowed to proceed for 20 hr. at 60°C, after adding 125 μl of 3M NaCl and overlaying with mineral oil. The single stranded DNA remaining, after removal of cell DNA by hybridization to L-2 cell DNA, was selected on a hydroxyapatite column previously equilibrated with 0.12 M PO_4 at 60°C. The eluate fractions containing ssDNA possessing the highest radioactivity were pooled and dialyzed against 3 mM EDTA at 4°C. The dialysate was mixed with 10 μl of tRNA (10 mg/ml) and Na Acetate was added to a final concentration of 0.1 M. The cDNA, precipitated overnight with 2 volumes of 95% ethanol at -20°C, was sedimented at 16,000 x g and the pellet resuspended in 1.0 ml annealing buffer containing 50% v/v deionized formamide, 3 x SSC (0.45 M NaCl, 0.045 M sodium citrate), 1 x Denhardt's buffer (Denhardt, 1966) (.02% BSA, 0.02% polyvinyl pyrolidone 360, 0.02% Ficoll), 1 mg/ml yeast RNA, 100

µg/ml alkali-sheared salmon sperm single stranded DNA, and 0.01 M Hepes (pH 7.4). The cDNA was stored at -20°C until required for dot blotting.

3.21 RNA Extraction and Dot Blotting.

Primary rat oligodendrocytes were seeded on 35 mm dishes at a density of 1.5 x 10^6 cells/dish and infected with JHMV at a m.o.i. of 1-2 PFU/cell. The cultures were monitored daily for the production of infectious progeny until a persistent infection had been established, whereupon RNA was extracted with guanidine-HCl for dot blotting (Cheley and Anderson, 1984). The RNA samples were applied to nitrocellulose (0.45 µm Schleicher and Schuell) in a series of 10-fold dilutions and baked to dryness at 80°C. Hybridization to [^{35}S] cDNA (approx. 10^6 cpm/blot) was carried out under stringent conditions at 42.5°C for 48 hr., then the blot was washed as described previously (Coulter-Mackie et al., 1980). The first step consisted of 5 x 5 min. washes at room temperature with 3 x SSC, the second step involved 4 x 15 min. washes at 50°C in 1 x SSC plus 0.1% SDS and the final step required 5 rinses in 0.1 x SSC at RT. The blots were then air-dried and wrapped in saran-wrap prior to autoradiography. Specificity of the probe was tested using JHMV infected L-cell RNA and purified JHMV RNA. The results were visualized on autoradiograms, using Kodak XAR-5 X-ray film.

3.22 Labelling of JHMV Proteins.

L-2 cells were seeded at a density of 3 x 10^6 cells/60 mm dish and incubated overnight at 37°C, then infected with JHMV at a m.o.i. of 1 PFU/cell. Spread of the infection was monitored until 60% of the cells had formed syncitia. The cells were then incubated in methionine-free medium or PO_4-free medium; containing 2% dialyzed FBS, until the infection had progressed to 100% syncitia formation. Labelling was carried out for 30 min. using [^{35}S] methionine, (NEN, > 800 Ci/mmole) or [^{32}P] ortho-phosphate (NEN, carrier free 10 mCi/ml), added at a final concentration of 100 uCi/ml. After labelling, the cultures were rinsed several times with ice-cold PBS, taken up in 200 µl of Tris-Triton and disrupted by repeatedly forcing the cells through a 30 gauge needle. Particulate matter was sedimented at 13,000 x g in an Eppendorf centrifuge at 4°C for 5 min. The supernatant containing the labelled viral proteins was transferred to a 500 µl-capacity Eppendorf tube and stored at -70°C.

3.23 Preparation of [^{32}P]-Labelled Casein.

Phosphorylation of casein for use as a phosphoserine substrate was carried out according to the method of Nelson and Branton (1984). Briefly, 500 ug of α-casein (Worthington) was incubated for 40 min. at 37°C in a 200 µl reaction volume containing 40 mM MES (pH 7.0), 10 mM $MgCl_2$, 7.5 ug of the catalytic subunit of beef heart cyclic AMP-dependent protein kinase (Sigma) and 150 uCi of γ-P^{32} ATP (NEN >3000 Ci/mmol). The reaction was terminated by addition of trichloroacetic acid (TCA) to a final concentration of 12%. Following precipitation

for 1 hr. on ice the precipitate was sedimented at 13,000 x g for 5 min. in an Eppendorf centrifuge. The pellet was resuspended in ice-cold acetone containing 2% (vol/vol) 1N HCl and resedimented. The pellet was resuspended in water and subjected once more to precipitation with TCA followed by the acetone-HCl wash. The material obtained, which was essentially free of [γ-^{32}P]ATP, was dissolved in 20 mM Tris-HCl buffer (pH 7.0) containing 0.25 M sucrose, 20 mM NaCl, and 2 mM 2-ME. Insoluble material was centrifuged into pellets at 13,000 x g for 10 min. and the supernatant, containing the phosphorylated casein, was stored at -20°C.

3.24 Assays for Phosphatase Activity.

Phosphatase activity was assayed according to Pallen and Wang (1983), using para-Nitrophenyl phosphate (pNPP) as substrate in a 1.0 ml reaction volume containing 25 mM Tris, 25 mM MES (pH 5.5), 2 mM pNPP, 1 mM $MnCl_2$ and 50 μg protein extract from cultured astrocytes or oligodendrocytes. The mixtures were incubated at 30°C for 20 min. and the reaction terminated by the addition of 50 μl, 13% K_2HPO_4. The samples were then clarified by centrifugation at 1,000 x g and the amount of pNPP substrate consumed was determined by measuring the absorbance of the supernatant at 410 nm.

Phosphoserine-specific phosphatase activity against JHMV nucleocapsid (NC) protein as the substrate was assayed in a 50 μl reaction volume containing 25 mM Tris, 25 mM MES (pH 5.5), 5 ul [^{35}S] or[^{32}P]-labelled JHMV infected L-2 cell extract, 1 mM divalent cation, and 50 μg of protein from the oligodendrocyte particulate fraction.

After incubation for 90 min. at 30°C the reaction was terminated by mixing 1:1 with sample buffer containing 8 M urea, 20% glycerol, 20% 2-ME, 20 µl acetic acid/10 ml sample buffer and 1 µl carbol fuschin. Following centrifugation for 5 min. in an Eppendorf centrifuge the samples were loaded immediately onto cylindrical gels (Mets and Bogorad, 1974) and subjected to polyacrylamide gel electrophoresis.

Phosphoserine-phosphatase activity, against α-casein as the substrate, was measured in a mixture containing 25 mM Tris, 25 mM MES (pH 5.5), 1 mM $FeCl_2:4H_2O$, 50 µg protein extract from the particulate fraction of oligodendrocytes and 5 µl of [^{32}P]α-casein. The mixture was incubated at either 30°C or 37°C and stopped at 2.5 min. intervals by snap freezing in liquid nitrogen (Nelson and Branton, 1984). Proteinaceous material was precipiated on ice overnight with 400 µl of 25% TCA and 50 µl of 1% BSA. Free phosphorous was partitioned into isobutyl alcohol according to the method of Maeno and Greengard (1972). Briefly, 400 µl of the deproteinized extract were mixed vigorously with 50 µl of 0.01 M KH_2PO_4 and 150 µl of 5% ammonium molybdate. The phosphomolybdate complex which was formed was extracted with 1.0 ml of isobutyl alcohol. The free [^{32}P] was measured in triplicate samples in a liquid scintillation counter after mixing 100 µl aliquots of the isobutly alcohol extract with 5 mls of Formula 947 (NEN) scintillation cocktail.

3.25 Two Dimensional Gel Electrophoresis of Nucleocapsid Protein.

NC protein and related products generated by the phosphoprotein phosphatase reactions were analyzed by two different procedures originally described by O'Farrell et al. (1975) and Mets and Bogarad (1971). Glass tubes, 130 x 2.5 mm inside diameter, were used for both procedures. The tubes were prepared by soaking overnight in nitric acid, followed by 3 washes in hot water and 3 rinses in deionized water. The cleaned tubes were dipped in 0.01% (v/v) column coat (Miles Laboratory) and dried for 2 hr at 120°C in a drying oven. Prior to pouring all gels the bottoms of the tubes were sealed with parafilm.

Gels were prepared for isoelectric focusing by the method of O'Farrell (1975) as modified by Essani and Dales (1979). Briefly, the isoelectric focusing (IF) gel mixture consisted of 9.5 M urea, 11.5% acrylamide (acrylamide-bisacrylamide 28.38/1.62), 2% NP40 (v/v), and 2% ampholytes (pH 3/10:pH 5/7 at 1:4). The urea was completely dissolved by gently warming the mixture in a 37°C water bath with constant swirling. To each 5.0 ml of gel solution 10 µl of 10% ammonium persulphate was added followed by a brief period of degassing after which 7 µl of TEMED was added to initiate polymerization. The gels were quickly poured into the tubes using a pasteur pipette, overlayed with 8 M urea and allowed to polymerize for 1 to 2 hr. The overlay solution was replaced by 20 µl of lysis buffer containing 9.5 M urea, 2% NP40, 2% ampholytes (1.6% pH 5/7 and 0.4% pH 3/10), and 5% 2-ME. The gels continued to set for an additional 2 hr after which the parafilm was removed and the tubes placed in a standard tube-gel

electrophoresis chamber. The lysis buffer was subsequently removed, samples loaded onto the gels and overlayed with 25 µl of sample overlay solution diluted 1:1 with deionized water consisting of 8 M urea, 1% ampholytes (0.8% pH 5/7, 0.2% pH 3/10). The tubes were then filled very carefully with the 0.02 M NaOH cathode solution (which had been extensively degassed prior to use) then the tank filled with the same. The lower chamber was filled with 0.01 M H_3PO_4 anode solution. The IF gels were run at a constant voltage under the following regimen: 200 V for 5 min, 300 V for 18 hr and 400 V for 90 min. Gels were extruded from the tubes using a 5 ml syringe fitted with Tygon tubing and filled with water. The gels were either stored in SDS sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 10% (w/v) glycerol, 5% (v/v) 2-ME, and 2.3% (w/v) SDS or equilibrated at 37°C for 20 min with 4 changes at 5 min intervals prior to loading onto SDS-PAGE gels.

Optimal resolution of basic proteins was achieved with the two-dimensional electrophoresis method described by Mets and Bogarad (1974). Reaction mixtures were electrophoresed in the first dimension for 10 hr. at a constant current of 1 mA/gel through a 4% polyacrylamide gel consisting of 4% acrylamide, 0.1% methylene bisacrylamide in a gel buffer containing 8 M urea and 0.057 M Bistris (Sigma) using a discontinuous buffer system. The upper gel buffer consisted of 0.01 M Bis-Tris (pH 4.0)and the lower gel buffer contained 0.179 M potassium acetate (pH 5.0). The pH of all buffers was adjusted by addition of glacial acetic acid. After completion of electrophoresis in the first dimension the gels were equilibrated in a

buffer containing 6.25 ml of upper gel buffer (Laemmli, 1970), 2.5 ml 10% SDS, 0.5 ml 2-ME, and 1.0 ml glycerol made up to 50 ml in H_2O for 20 min. with a fresh change of buffer every 5 min.

Following equilibration both these and IF gels were positioned horizontally on 10% SDS-polyacrylamide slab gels (SDS-PAGE) (Laemmli, 1970), sealed with 1% agarose dissolved in upper gel buffer and electrophoresed in the second dimension at 35 mA/gel, until the tracking dye was approximately 5 mm from the bottom of the plates. Gels were dried under vacuum and the NC related material visualized on autoradiograms produced with Kodak XAR-5 X-ray film.

3.26 Two Dimensional Tryptic Peptide Mapping.

[^{32}P]-labelled NC material was prepared for two dimensional tryptic peptide mapping according to the procedure outlined by Zweig and Singer (1979). Spots related to NC and presumed NC products were excised from dried 2-D PAGE gels, then transferred to siliconized tubes containing 1 ml of 0.05 M NH_4HCO_3 (pH 8.0) and 50 µg of trypsin pretreated with TPCK (Sigma). Following incubation for 24 hr. at 37°C the hydrolyzed peptides were transferred again to clean siliconized tubes, lyophilized and resuspended 2-3 times in 1 ml H_2O. The final lyophilysate was resuspended in 10 µl of electrophoresis buffer (pH 1.9) consisting of acetic acid:formic acid:H_2O, 15:5:80. The phosphopeptides were separated on 10 cm x 10 cm thin layer cellulose plates (Merck) by electrophoresis at 1000V for 20 min., followed by ascending chromatography in one of 2 solvent systems consisting of either 1-butanol:pyridine:acetic acid:H_2O, 32:5:25:5:20 for 3 hrs. or

isobutytic acid:pyridine:acetic acid:butanol:water, 65:5:3:2:29 (Scheidtmann, 1986) for approximately 1.5 hr. The phosphopeptides were visualized by autoradiography using Kodak XAR-5 X-ray film.

3.27 Labelling of JHMV RNA.

Endogenously [^{32}P]-labelled JHMV RNA was prepared as described previously (Robbins et al., 1986), however, the RNA was extracted with guanidine-HCl, as described elsewhere (Strohman, et al., 1977). A 100 mm confluent L-2 cell culture was inoculated with JHMV at a m.o.i. of 5 PFU/cell. At 1.5 hr. post inoculation the medium was replaced with PO_4-free medium with Earle's salts (Gibco), supplemented with 2% dialyzed FBS and 5 µg/ml actinomycin D. At 2.75 hr. post infection [^{32}P]- orthophosphate (NEN, 10 mCi/ml) was added to a final concentration of 150 µCi/ml. When the infection had spread to involve all cells in the formation of syncitia, the culture was rinsed 3-4 times with ice-cold PBS, then solubilized in 3 ml of guanidine-HCl. The cells were disrupted by forcing the suspension five times through a 21 gauge needle and the RNA precipitated by adding 0.6 volumes of 95% ethanol and allowing the mixture to stand at -20°C overnight. The precipitated RNA was extracted again and precipitated once more. Following centrifugation the final RNA pellet was resuspended in 100 µl of 0.01%, diethylpyrocarbonate-treated, deionized H_2O and stored at -20°C.

3.28 Nucleic Acid Overlay-Protein Binding Assay.

Protein blots were preparared by electroelution from 7.5-15% SDS-PAGE gradient gels onto a 0.22 μm nitrocellulose matrix (Schleicher and Schuell) (Bowen et al., 1980) at 50 V for 12-16 hr. at 4°C. The transfer buffer consisted of 25 mM Tris, 192 mM glycine (pH 8.3) with 20% methanol (Robbins et al., 1986). Following transfer, the proteins were visualized by staining with 0.01% amido black (Towbin et al., 1979). Individual strips were cut from the nitrocellulose corresponding to the lane of each sample. Strips could be stored in transfer buffer at 4°C (Robbins et al., 1986) or used at once for the nucleic acid overlay-protein binding assay (NOPBA).

The strips were immersed in standard binding buffer (SBB) containing 0.05 M NaCl, 1 mM disodium EDTA, 10 mM Tris-HCl (pH 7.0), 0.02% BSA, 0.02% polyvinyl pyrrolidone (PVP-40), and 0.02% Ficoll for 30 min. prior to reacting with the nucleic acid probes (Bowen et al., 1980; Robbins et al., 1986). Following 2-3 rinses with SBB, strips were placed in 2 ml. of SBB containing either [^{32}P]RNA or [^{35}S]cDNA at 2.5 x 10^5 cpm/lane or 1.35 x 10^5 cpm/lane, respectively. The strips were incubated for 1 hr. with the probes at room temperature using constant rocking, then were washed 3 times for 15 min. with SBB, rinsed several times with SBB, air dried and subjected to autoradiography, employing intensifying screens.

CHAPTER 4

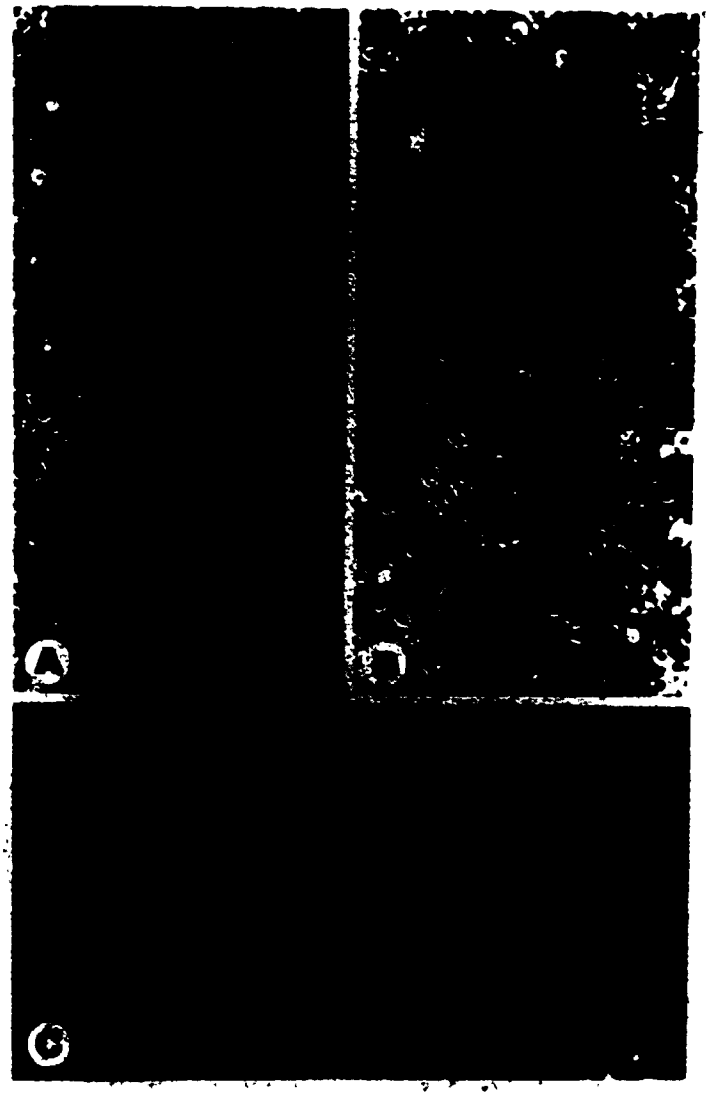
RESULTS

4.1 Comparison of MHV Replication and the Influence of Cell Density in Mixed and Selected Cultures from the CNS

The ability of MHV3 and JHMV to produce infectious progeny in freshly explanted mixed and shaken cultures was tested for the duration of each of the experiments described. Under the light microscope, the appearance of living mixed and selected primary brain cultures is illustrated in Figures 1, A-C. Judging primarily on morphological criteria, cultures enriched in oligodendrocytes and astrocytes were over 99% pure. Examination of mixed brain cultures under phase-contrast optics revealed the presence of a monolayer of subjacent astrocytic-type cells and a covering layer of scattered, refractile smaller cells with associated extensive processes (Fig. 1A). Oligodendrocytes appeared as small, highly refractile cells with extensive, arborizing processes (Fig. 1B). Astrocytes appeared as tightly adhering, angular, very flat cells of low phase density. Following cytoplasmic staining with specific R > GFAP antibodies the astrocytes displayed bundles of filaments characteristic of astrocyte GFAP (Fig. 1C), (Manthorpe et al., 1979).

After inoculating mixed cultures with MHV3 at m.o.i. of 0.1 to 1.0 PFU/cell, progeny virus could be detected within 12 hr. Subsequently, the titre progressively increased and remained at the high level for 4 to 5 weeks (Fig. 2A, Table 1). There was a coincidental, slowly developing CPE of the subjacent astrocytic cells which became almost complete at the time virus production ceased. The

Figure 3 Immune labelling of oligodendrocytes with antibodies directed against oligodendrocyte specific antigens. A. Mixed glial cell culture containing oligodendrocytes and astrocytes. Oligodendrocytes are identified by labelling for the surface specific galactocerebroside with R>GC (C). B. Image of a cell with typical oligodendrocyte morphology at 15 days post explantation. The cell is identified as an oligodendrocyte by staining for the cytoplasmic specific marker myelin basic protein with R> MBP (D).


A
B
C

CPE, in the form of syncytia, was clearly evident under phase-contrast optics within 1 to 2 weeks following inoculation. The covering cells of presumptive oligodendrocytes were, by contrast, unaffected.

Inoculation of mixed cultures with m.o.i. of 0.1 to 1.0 PFU/cell of JHMV gave variable data, depending on the cell density. With dense cultures, plated at 5 X 10^5 cells/cm^2, progeny were detected within 12 hr, virus yields became maximal at 10^5 to 10^6 PFU/ml within 2 to 3 days and continued for prolonged periods, frequently for 3 to 4 weeks (Table 2, Fig. 2B). Thereafter, production ceased abruptly. When sparsely seeded cultures, containing approximately 1 X 10^5 cells/cm^2 were infected with JHMV at m.o.i. of 0.1 to 1.0 PFU/cell, there was only a transient burst of virus production, commencing usually at 12 hr., reaching a peak of only 10^2 PFU/ml and then ceasing (Fig. 2A). These observations revealed the close relationship between duration of JHMV replication and the density of primary rat cerebral cell explants, a relationship which did not occur with MHV3 in astrocytes. Cell density-dependence of JHMV replication in explanted oligodendrocytes will be considered further, below.

Another general feature of the replication process of CV in primary rat brain cultures is thermosensitivity, whereby formation of infectious progeny is arrested at 39-40°C, the non-permissive temperature. However, contrary to the situation with continuous neural and other rat cell lines, in which restriction at 39.5°C is complete (Lucas et al., 1978), in the case of primary brain cultures examined here the temperature restriction was found to be incomplete (Fig. 2). As in the case of the continuous rat schwannoma RN-2 line,

Figure 2 Replication of JHMV (▲) and MHV3 (●) in low density mixed cultures (A) and high density mixed cultures, (B) at the permissive (32.5°C for MHV3, 37°C for JHMV) and restrictive (39.5°C) temperatures. Arrows indicate times of temperature shifts. The m.o.i. in each experiment was approximately 0.1 PFU/cell.

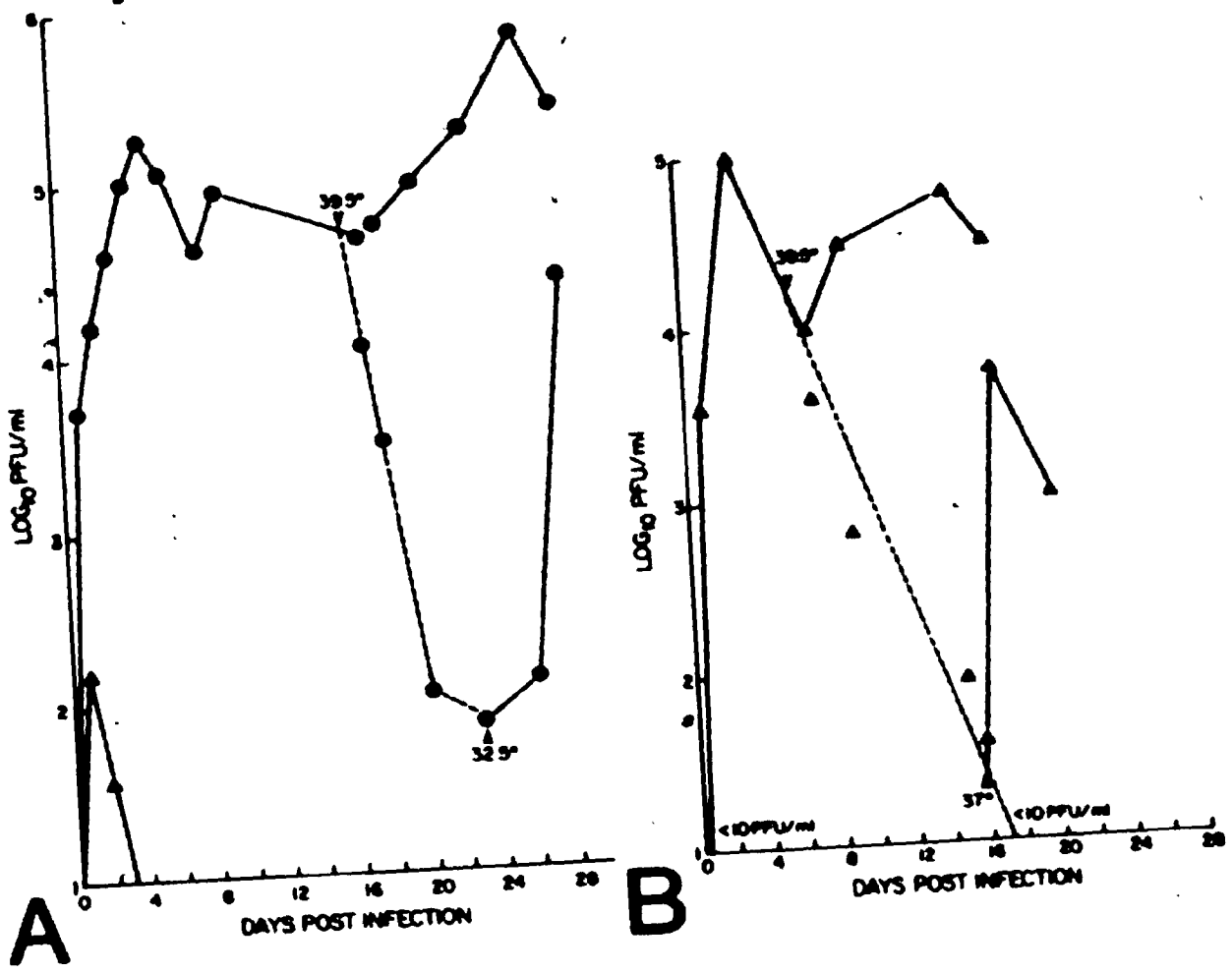

A
LOG10 PFU/ml
DAYS POST INFECTION
39.5°
32.5°
B
LOG10 PFU/ml
DAYS POST INFECTION
39.0°
37°
<10 PFU/ml
<10 PFU/ml

Table 1

In Vitro Replication of Coronaviruses in Low Density Mixed or Shaken Rat Cortical Cell Cultures

Days Post	Mixed Cultures		Shaken Cultures	
Inoculation	MHV3	JHMV	MHV3	JHMV
1	10-100	1-10	0.1-1	1-10
2	100	1	0.1	1
4	100-1000	<0.1-1	<0.1	<0.1
8	100-10,000	<0.1	<0.1	<0.1

All titers expressed X 10^2 PFU/ml.

Values represent the range of titres observed from not less than three separate experiments.

(Lucas et al., 1978), temperature shift-down from 39.5 to 32.5°C allowed, within the specified period of the experiment, the resumption of replication.

To ascertain the specificity of the tropism of CV serotypes for different cell types from rat brain, separate, relatively pure oligodendrocytic and astrocytic cultures were established as described in Materials and Methods and challenged with JHMV or MHV3. Inoculation with MHV3 of the astrocytes resulted in formation of infectious particles which simulated that described in the case of mixed cultures (Table 1, Fig. 2A). By contrast, infection of oligodendrocytic cultures with MHV3 (data in Table 1) and of astrocyte cultures with JHMV (not shown) at m.o.i. of 0.1 to 1.0 PFU/cell did not lead to any virus production. However, infection of oligodendrocyte cultures with JHMV at the same m.o.i.'s induced replication which, as with the mixed cultures, was either transient or persistent, depending on cell density (Table 2). More specifically, persistent infection could be established routinely in shaken cultures, hereafter termed oligodendrocytes, when seeded at a density greater than 2×10^5 cells/cm^2. With sparser cultures, at or below 1×10^5 cells/cm^2, JHMV was replicated only transiently and to low titre, regardless of the m.o.i. employed (Table 2, Fig. 2a). Thus, cultures seeded at 5×10^5 cells/cm^2 produced a maximum of approximately 10^6 PFU/ml, those seeded at 2×10^5 cells/cm^2 approximately 10^3 to 10^6 PFU/ml and those at 10^5 cells/cm^2 or less only about 10^2 to 10^4 PFU/ml depending on the moi. used to initiate infection. Evidently the yields, in terms of PFU/cell, were not in

Table 2

Effect of Cell Density on the Replication of JHMV in Primary Oligodendrocytes

Cell Density	M.O.I.	Days Post Infection 1	2	3	6	8	10	15
Medium[a]	5	100	1000	500	120	100	> 10,000	180
	1	33.6	1000	668	488	900	> 10,000	30
	0.1	2.6	42.6	62	800	944	> 10,000	30
Low[b]	12.5	28.8	80	10	2.6	<0.1	<0.1	<0.1
	2.5	10.4	30.8	10	1.2	0.8	0.16	<0.1
	0.25	0.6	3	0.64	1.08	0.08	<0.1	<0.1

All titers expressed X 10^2 PFU/ml.

a) approximately 2.5 X 10^5 cells/cm^2

b) approximately 1 X 10^5 cells/cm^2

Values represent titres obtained from a single experiment that corroborate the trend observed in a previous experiment.

direct proportion to the cell number but were related to increased cell to cell contact. Furthermore, the rapidity of the CPE manifested was also directly related to cell density, being detectable earlier and with greater frequency in the denser cultures.

4.2 Identity of the Cell Type in Shaken Cultures Replicating JHMV

Once it had been established that JHMV could persistently infect shaken cultures it was essential to ascertain whether these cells were, indeed, oligodendrocytes. For this purpose, indirect immunolabelling was carried out to associate the presence of viral antigen(s) with markers for the oligodendrocytes. Characterization of oligodendrocytes was conducted employing monospecific R>GC antisera and R>MBP antisera. It was observed that the majority, over 70 percent, of the cells were GC positive and, therefore, oligodendrocytes (Fig. 3). For correlating the presence of oligodendrocyte-specific and viral antigens within the same cells, fixation and permeation with acetone permitted simultaneous labelling with two specific antisera and different fluorochromes. The oligodendrocyte-specific cytoplasmic antigen, myelin basic protein (MBP), was detected with R>MBP monospecific serum. JHMV antigen(s) were detected by polyclonal antibodies raised in mice, as described in Materials and Methods. The data, illustrated in Figure 4, B,C revealed conclusively that MBP and viral antigens coexisted when the oligodendrocyte cultures were established at medium cell density and examined 20 days post explantation and 10 days after infection. When oligodendrocytes were seeded at low cell density and examined at 10

Figure 3 Immune labelling of oligodendrocytes with antibodies directed against oligodendrocyte specific antigens. A. Mixed glial cell culture containing oligodendrocytes and astrocytes. Oligodendrocytes are identified by labelling for the surface specific galactocerebroside with R > GC (C). B. Image of a cell with typical oligodendrocyte morphology at 15 days post explantation. The cell is identified as an oligodendrocyte by staining for the cytoplasmic specific marker myelin basic protein with R> MBP (D).

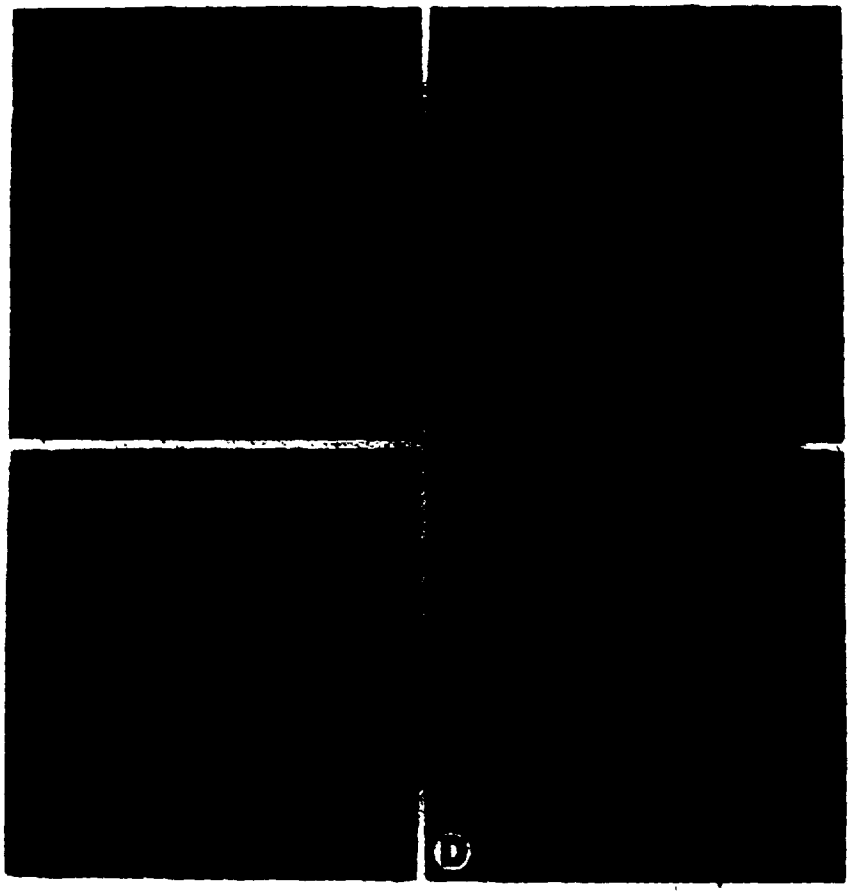
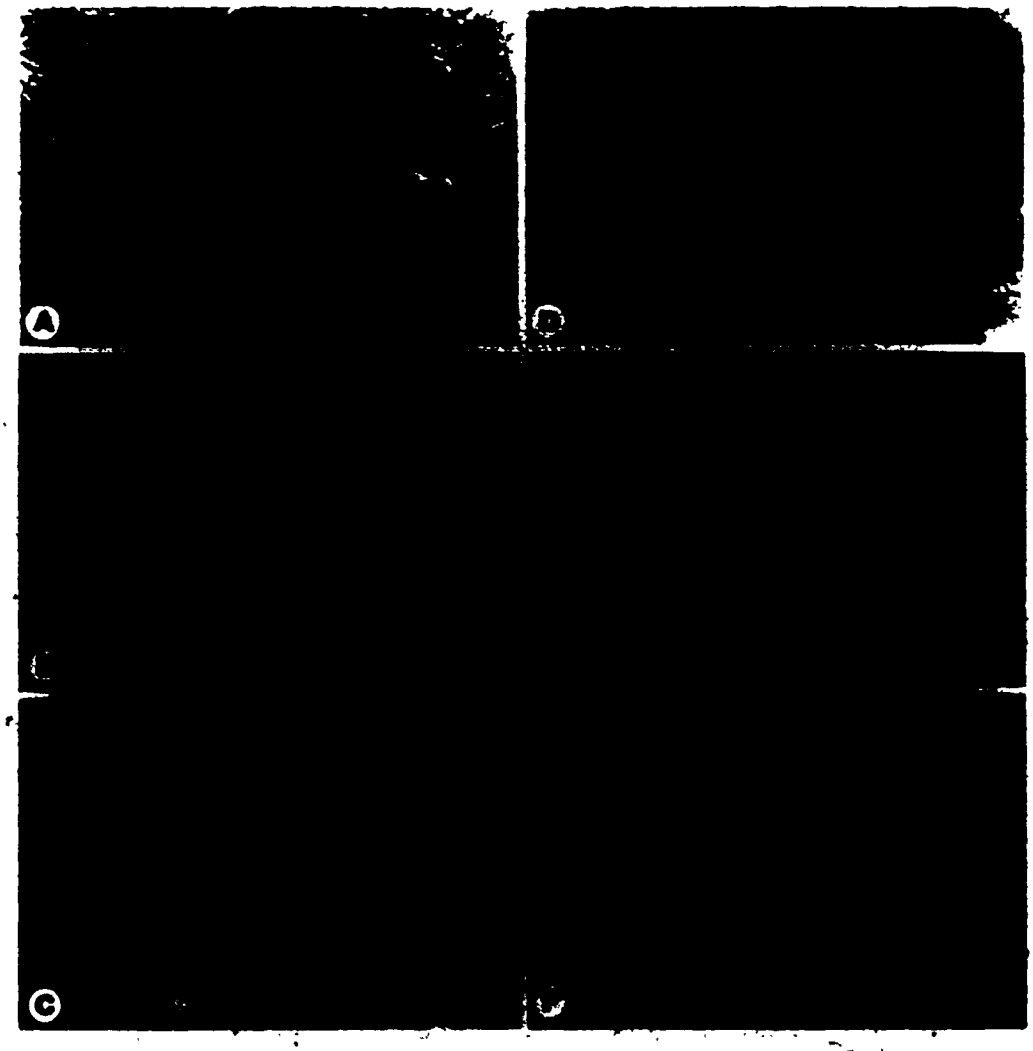

D

Figure 4 Images of JHMV infected oligodendrocytes at medium (left panel) and low density (right panel). A and D show phase images of virus infected cells, indicated by arrowheads. B and E are immunofluorescent images of cells expressing virus antigen while C and F demonstrate cells positive for myelin basic protein. The arrowheads in B and C depict a virus antigen negative, MBP positive cell while the arrowheads in E and F show a cell that stains for virus antigen but not MBP. X 3000.


A
B
C
D

days post explantation and 2 days after inoculation, virus antigen occurred with equal frequency within cells that were either MBP positive or negative (Fig. 4 E,F). This implies that younger cultures contained fewer cells expressing MBP, possibly in an earlier stage of differentiation, consistent with Barbarese et al (1981) who showed MBP to be a differentiation marker for oligodendrocytes in vitro.

Further characterization of virus-producing cell types was carried out at the fine structure level using transmission electron microscopy. Sections of JHMV infected material from shaken cultures revealed the presence of coronavirus particles in cells with the typical morphology of oligodendrocytes (Fig. 5), (Mori and LeBlond, 1970). By constrast, a survey of astrocyte cultures productively infected with MHV3 demonstrated particles characteristic of CV in cells containing numerous bundles of intermediate filaments, presumably GFAP, diagnostic for the astrocyte (Fig. 6) (Mori and LeBlond, 1969).

An independent approach towards identification of the cell type permissive or restrictive for JHMV was by means of immune lysis, utilizing surface specific antibody and complement. For this purpose the cells in oligodendrocyte cultures were inundated with R>GC antibody plus complement, then monitored 24 hr. after treatment for production of infectious progeny. A summary of the data, in Table 3, clearly shows that this treatment suppressed virus production, whereas in the appropriate controls virus formation persisted. When checked at 3 and 5 days following immune lysis, the oligodendrocyte cultures did not resume JHMV production. Additional controls, using astrocytes

Figure 5 Electron micrograph of an oligodendrocyte infected with JHMV. Dark staining cytoplasm, eccentric nucleus, lack of intermediate filaments, and an abundance of microtubules are used to identify oligodendrocytes ultrastructurally. (Mori and DeBlonde, 1970). Note the appearance of JHMV particles within cytoplasmic vesicles in the area of the endoplasmic reticulum and Golgi, as indicated by small arrow heads. X 660. Inset: large arrowheads point to budding of JHMV into cisternae typical to the morphogenesis of coronaviruses. X 54,000.

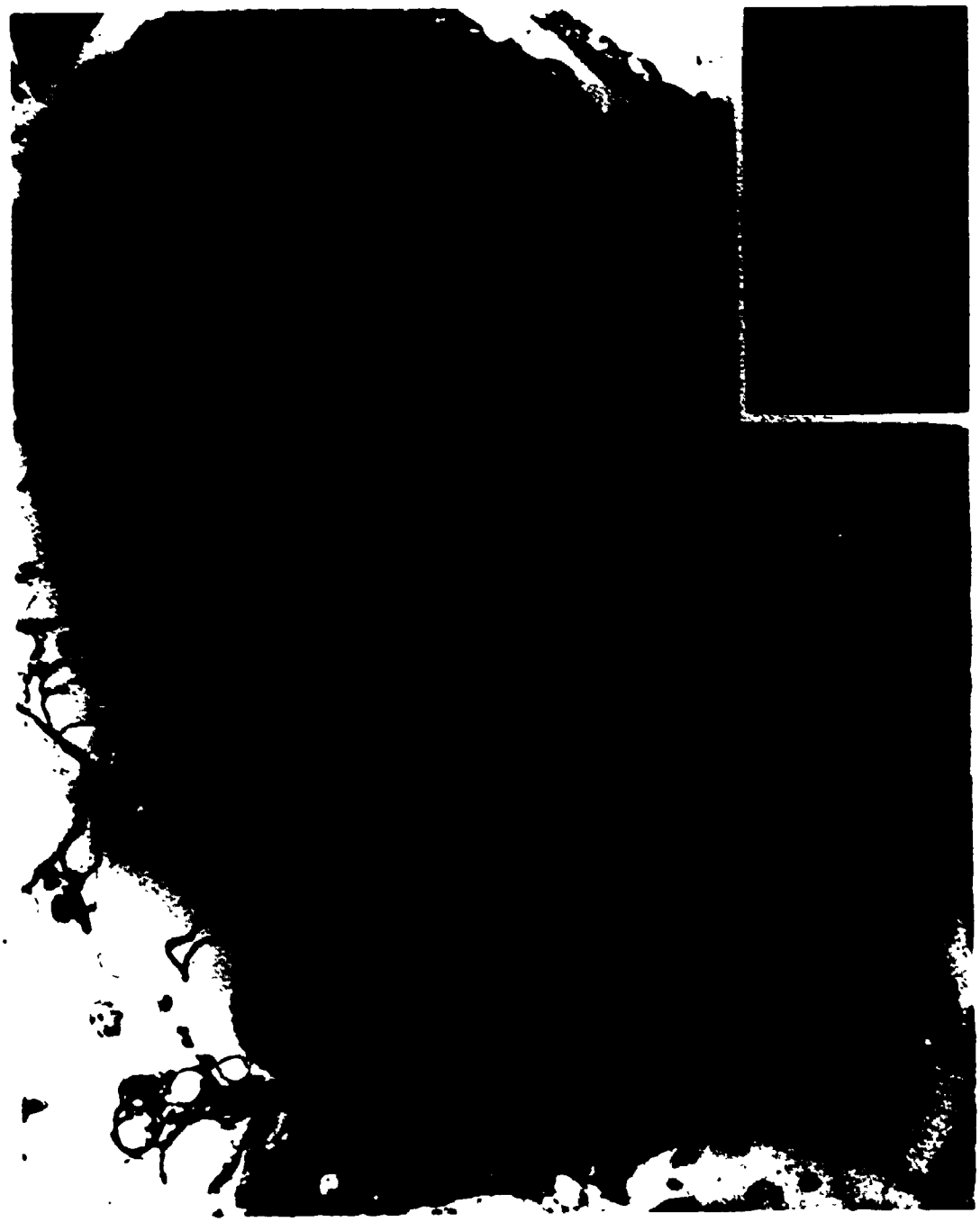


Figure 6 Electron micrograph of an MHV3 infected astrocyte. Astrocytes can be identified ultrastructurally by an abundance of intermediate filaments (F) and relatively light staining cytoplasm (Mori and LeBlond, 1969). Inclusions of viral nucleocapsids (NC) can be seen throughout the cytoplasm of infected cells. Mature viral particles can be found in the cisternae of endosplasmic reticulum and in other extracellular spaces, as indicated by arrowheads. X 21,000.

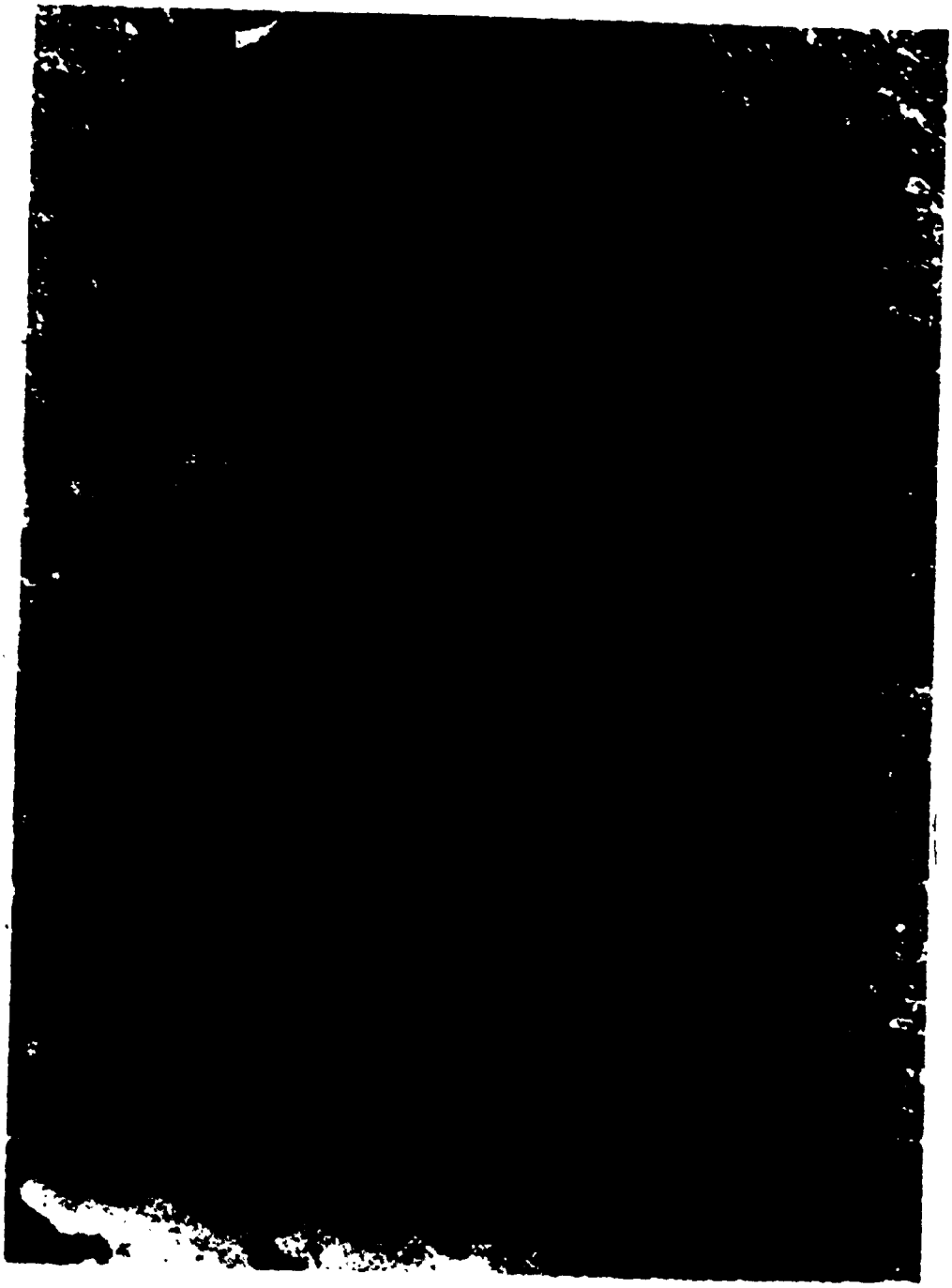


Table 3

Effect of Immune Lysis on the Replication of JHMV in Primary Oligodendrocytes

Treatment of Culture	Virus Produced
Oligodendrocytes Infected with JHMV	80
Complement Only	60
Anti-GC Antibody Only	60
Complement + Anti-GC Antibody	0*
Astrocytes Infected with MHV3	1.8
As Above Complement + Anti-GC Antibody	1.2
LMTK$^-$ Mouse Fibroblasts Infected with JHMV	> 1000
As Above Complement + Anti-GC Antibody	> 1000

*Most of the cells were lysed.

All titres expressed x 10^2 PFU/ml

Values represent titres obtained from a single experiment that corroborate the trend observed in a previous trial.

infected with MHV3 and JHMV chronically infected LMTK$^-$ cells (Mizzen et al., 1983), showed that R>GC antibody and complement did not lyse these cells and suppress the infections. On the basis of these observations it was concluded that *in vitro* tropism of JHMV is exclusive for oligodendrocytes and of MHV3 for astrocytes in CNS explants from the rat.

4.3 Influence of Oligodendrocyte Differentiation on JHMV Replication

Age-related differentiation of oligodendrocytes has been shown to be correlated *in vivo* (Sprinkle et al., 1978) and *in vitro* (McMorris, 1983) with increases in the levels of intracellular cAMP and CNPase, the enzyme marker for myelin synthesis. To ascertain whether oligodendrocyte differentiation also influenced virus replication, oligodendrocytes were assayed for CNPase and challenged with JHMV at intervals following explantation. Data, summarized in Table 4 show that enzyme induction was maximal by the 15th day and remained at a high level beyond the 21st day, in general agreement with results reported previously (Sprinkle et al., 1978; McMorris, 1983). JHMV replication was reproducibly suppressed sometime between the 15th and 21st days. With VSV, by contrast, there was no evidence of an age related inhibition of replication. Another approach for testing the relationship between differentiation and JHMV production was to treat oligodendrocytes with dbcAMP either 48 hr., prior to or following inoculation. The data, summarized in Table 5, revealed that cultures treated 48 hr. prior to inoculation failed to replicate the virus, whereas cultures treated after inoculation continued to produce

Table 4

Correlation Between CNPase Activity and Virus Replication in Shaken Cultures

Enzyme Activity and Virus Yield	Days Post Explantation 10	15	21
CNPase Activity nmoles/min/mg protein	679	1820	1790
JHMV PFU/ml	11.1	14.5	0.3
VSV PFU/ml	155	735	440

All titres expressed X 10^2 PFU/ml

Titres monitored 24 hpi.

Values represent data obtained from a single experiment corroborating the trend observed in two previous trials.

Table 5

Effect of N^6, $O^{2'}$-Dibutyryl 3':5' Cyclic AMP on the Replication of JHMV in Primary Oligodendrocytes

	Days Post Infection						
Treatment of Culture	2	3	4	6	8	12	14
Control	13	46.8	55	38.8	720	31	20
1mM Post Infection	33.4	100*	15.5	100	500	26	30
1mM 48 hours Before Infection	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
1mM 48 hours Before Infection	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

All titres expressed X 10^2 PFU/ml

* dbcAMP added

** dbcAMP removed

Values represent data obtained from a single experiment corroborating the trend observed in a minimum of three previous trials.

progeny. Data, presented in Table 6, reveal that both 1mM dbcAMP and 7μM papaverine, (the latter being an inhibitor of cAMP phosphodiesterase which indirectly causes the accumulation of intracellular cAMP), were effective in repressing JHMV formation in oligodendrocytes seeded at medium density. It may be highly significant that cell density has a profound influence on the efficacy of dbcAMP. Thus, unlike medium density cultures, cells seeded at the high density were unaffected by 1mM dbcAMP but were influenced by 5 and 10 mM dbcAMP with respect to suppression of virus replication (Table 6). These data are supported by the findings of Wernicke and Volpe (1986), who were able to demonstrate the effects of cell density in oligodendrocyte differentiation whereby the temporal expression of enzymes characteristic of oligodendroglial differentiation were greatest in cultures of low cell densities. By comparison, temporal regulation of astrocyte differentiation was shown to be unaffected by cell density.

To test for the production of viral antigens in the oligodendrocytes, cultures pretreated with 1 mM dbcAMP and 7 μM papaverine were tested 24 hours after infection with M>MHV antibodies by means of indirect immunofluorescence. The results indicated that JHMV related antigens were present in the cytoplasm of untreated oligodendrocytes (Fig. 4) but not in treated cells (not shown).

Table 6

Effect of Differentiation Inducers and Cell Density on the Replication of JHMV in Primary Oligodendrocytes

Conditions of Culture[a]		Control	Treated
Medium Density	1mM dbcAMP	50	0.6
	7uM Papaverine	50	8.4
1mM dbcAMP infected with VSV		30,000	35,500
High Density	0.1 mM dbcAMP	> 1,000	> 1,000
	0.5 mM dbcAMP	> 1,000	> 1,000
	1.0 mM dbcAMP	> 1,000	> 1,000
	5.0 mM dbcAMP	> 1,000	18
	10.0 mM dbcAMP	> 1,000	1
LMTK$^-$ Mouse Fibroblasts		8400	5300
	1.0 mM dbcAMP		

All titres expressed X 10^2 PFU/ml

a) M.O.I. of 1.0 in each case.

Values represent data obtained from a single experiment corroborating the trend observed in two previous trials.

To determine whether the effects of dbcAMP were specifically related to the CV, medium density cultures were challenged with VSV and MV. Both agents were replicated with equal efficiency, regardless of the treatment imposed upon the oligodendrocytes, (data on VSV in Table 6, on MV not shown).

Virus replication in LMTK$^-$ cells, also used as a control, was affected only marginally by exposure to dbcAMP (Table 6).

4.4 Relationship Between Intracellular Levels of cAMP and Virus Replication

Metabolites which affect intracellular levels of cAMP have been shown to profoundly affect virus replication in various cell types (Robbins and Rapp, 1980; Miller and Carrigan, 1982; Van Alstyne and Paty, 1983; Yoshikawa and Yamanouchi, 1984; Beushausen and Dales, 1985). The effect may be on either activation of the production of infectious particles of a virus, formerly in a latent state (Van Alstyne and Paty, 1983), or suppression of formation of infectious progeny (Robbins and Rapp, 1980; Miller and Carrigan, 1982; Yoshikawa and Yamanouchi, 1984; Beushausen and Dales, 1985). In the case of MV infection of neural cell lines, cAMP-induced repression is reversible upon removal of the metabolite (Robbins and Rapp, 1980; Miller and Carrigan, 1982). However, with CV it has been demonstrated that elevation of cAMP concentration, which is coincident with differentiation, causes an irreversible block of replication of CV in primary rat and mouse oligodendrocytes (Beushausen and Dales, 1985; Wilson et al 1986).

In addition to dbcAMP and papaverine the effects of treatment with a second analogue of 3':5' cAMP, known to enhance differentiation in primary and continous cell lines, was examined with respect to the formation of infectious CV progeny. As evident in Table 7, pretreatment with 1mM dbcAMP entirely inhibited the replication of JHMV in rat oligodendrocytes, confirming previous findings (Beushausen and Dales, 1985). The analogue 8-Br cAMP, an inducer of differentiation in cultured rat schwann cells (Sobue and Pleasure, 1984; Sobue et al, 1986), and oligodendrocytes (Kim et al, 1985; Pleasure et al, 1986) also precluded virus production in cultured primary oligodendrocytes.

Compounds which can bring about an increase of the intracellular cAMP concentration through their indirect action on the adenylate cyclase system were also tested for their ability to repress CV replication in primary rat oligodendrocytes. One of these substances, isoproterenol, a catecholamine which binds specifically to B adrenergic receptors, increases the CNS concentration of cAMP through activation of the adenylate cyclase system, which results from receptor coupling to the GTP binding protein, Gs (Gilman, 1984). The data in Table 7 demonstrate that 50 uM of isoproterenol completely inhibited JHMV replication in oligodendrocytes.

The diterpene, forskolin, which by direct interaction with the adenylate cyclase system causes increases of intracellular cAMP levels (Metzger and Lindner, 1981; Seamon and Daly, 1981; Seamon et al., 1981) also suppressed formation of JHMV in oligodendrocytes. Treatment at several concentrations showed that the effect was

Table 7

Effect of Differentiation Inducers on Coronavirus Replication in Primary Rat Oligodendrocytes

Treatment	Days After Infection		
	1	3	10
Control	1.0	1.0	100
1mM dbcAMP	<0.1	<0.1	<0.1
1mM 8-BrcAMP	<0.1	<0.1	<0.1
20μM Isoproterenol	1.75	<0.1	500
50μM Isoproterenol	0.15	<0.1	<0.1
20μM Forskolin	0.3	0.05	70
50μM Forskolin	0.25	<0.1	<0.1
100μM Forskolin	0.8	<0.1	<0.1

Note: All titres x 10^2 PFU/ml

Cell density: 2-3 x 10^5 cells/well

*sampled 9 days after infection

Values represent data compiled from several unrelated experiments. However, trends were observed to be consistent for each treatment.

Table 8

Effect of dbcGMP on the Replication of JHMV

in Primary Rat Oligodendrocytes

Treatment	Days After Infection	
	1	2
Control	85.8	158
1mM dbcGMP	99.7	112
1mM dbcAMP	<0.1	<0.1

Note: All titres x 10^2 PFU/ml

Cell density: 4-5 x 10^5/well

Values listed represent the average of two samples per experiment and corroborate the trend observed in three such trials.

complete at 50 μM (Table 7). It should be pointed out that minimum concentrations of isoproterenol and forskolin required to repress virus replication in oligodendrocytes are approximately 5 fold greater than those reported to be necessary for activating the adenylate cyclase system. It is presumed that in oligodendrocyte cultures there is a relationship between the concentration of metabolite added and the rate of adenylate cyclase induction, hence intracellular accumulation of cAMP. The validity of this assumption has been demonstrated with forskolin in rat cerebral cortical membranes (Seamon and Daly, 1981), cultures of S_{49} mouse lymphoma cells (Barber and Goka, 1984), and primary rat Schwann cells (Sobue et al., 1986).

It has been suggested that aside from cAMP and calcium, cGMP acts as an important second messenger for regulating neuronal functions through the activation of cGMP-dependent protein kinase (Nestler and Greengard, 1983). To test the possibility that elevation of cyclic nucleotide concentrations in general, and of cGMP in particular, may suppress CV replication we treated oligodendrocytes with dbcGMP prior to infection with JHMV. It is evident from the data in Table 8 that dbcGMP was not inhibitory towards JHMV, indicating that CV suppression in oligodendrocytes is specifically related to metabolic events affected by cAMP levels.

It should be noted that there is an apparent disparity between data in Tables 7 and 8 concerning the PFU/ml of JHMV produced within 24 hr. after infection of the untreated oligodendrocytes. These

differences in titre are attributed to the minor changes in density of oligodendrocytes in culture which were shown previously to profoundly influence JHMV production (Beushausen and Dales, 1985).

Infection of rat neural cells by CV is characterized by an unambiguous tropism of strain MHV3 for astrocytes and JHMV for oligodendrocytes (Beushausen and Dales, 1985). In this system it was demonstrated that a 48 hr. pretreatment of oligodendrocytes with dbcAMP completely abolished JHMV replication, whereas it only marginally affected the replication of MHV3 in astrocytes. To determine whether a prolonged exposure of astrocytes would be more effective, primary astrocyte cultures were pretreated with 1 mM dbcAMP and the titres of MHV3 produced determined. It is evident from Table 9 that pretreatment of astrocytes for as long as 5 days did not diminish the ability of these cells to produce virus. This observation is consistent with the view that elevation of cAMP affects differentially CV replication in astrocytes and oligodendrocytes.

To examine whether cAMP influences CV replication in other cell types in which differentiation can be induced, L6 rat myoblasts and the L6 mutant subline, JRU_5 were exposed to 1 mM dbcAMP. It is evident from the results in Table 10 that pretreatment of both types of myoblasts arrested production of MHV3 and JHMV. By comparison, the untreated controls became persistently infected, yielding within 3 days titres in excess of 10^3 PFU/ml, in agreement with Lucas et al. (1978). It is probably not coincidental that treatment with dbcAMP affects CV production in myoblasts and oligodendrocytes in a similar manner, because it has been shown that drugs which stimulate the

Table 9

Effect of Prolonged Exposure to dbcAMP on the Replication of MHV3 in Primary Rat Astrocytes

Treatment	Days After Infection	
	3	5
Control	3.5	1.1
1 day, 1mM dbcAMP	1.6	1.7
3 days, 1mM dbcAMP	1.6	2.0
5 days, 1mM dbcAMP	1.6	3.2

Note: All titres x 10^2 PFU/ml

Values listed represent data obtained from a single experiment and corroborate the trend observed in a previous trial.

formation of cAMP in myoblasts (Zalin, 1977) also promote differentiation somewhat akin to the in vitro maturation of neonatal brain cells into oligodendrocytes.

4.5 Responses of cAMP-Dependent Protein Kinases to Modulation of cAMP Concentration

It is now well established that cAMP, acting as a second messenger, participates in a variety of cellular functions, among them those related to hormonal effects and the regulation of gene expression (Cohen, 1982; Jungmann et al., 1983; Kondrashin, 1985; Nagamine, 1985)). Increases in intracellular levels of cAMP produce changes in the enzymatic activities of two cAMP-dependent protein kinases, PKI and PKII, which are involved in initiating the phosphorylation of various intracellular substrates (Krebs and Beavo, 1979; Cohen, 1982). In the nervous system, the role of cAMP is presumed to be fundamental for neurotransmission and neuronal/glial cell communication (Gilman and Nirenberg, 1971; Schramm and Zelinger, 1984), two functions generally presumed to be mediated through the activation of PKII, the predominant PK activity in brain tissue (Nairn et al., 1985; Weldon et al., 1985). The relative activities of PKI and PKII in untreated neural cell material were first assessed prior to examining the effects of modulating cAMP levels. For this purpose, cell free extracts of astrocytes and oligodendrocytes were prepared, then separated into fractions by DEAE-cellulose column chromatography, and assayed for [^{3}H]-cAMP binding and kinase activity. It is evident from the data in Figure 7 that neural cells were conspicuously devoid

Figure 7 Chromatography of cAMP dependent protein kinases and respective regulatory subunits in primary neural cells. The cytosol (40,000 x g) component of astrocytes (A) and oligodendrocytes (B) was prepared and separated into 1 ml fractions on DEAE-cellulose, as described under Materials and Methods, then assayed for cAMP dependent protein kinases, PKI and PKII (●). The data were calculated as the difference between values obtained in the presence and absence of 1.5 μM cAMP. The regulatory subunits, RI and RII (○) were detected by binding to [^{3}H] cAMP as described under Materials and Methods. The products were eluted by means of a gradient (△) ranging from 0-400 mM NaCl in Buffer A. Arrows indicate peak fractions of PKI, RI and PKII. All activities were normalized for 100 mg protein loaded.

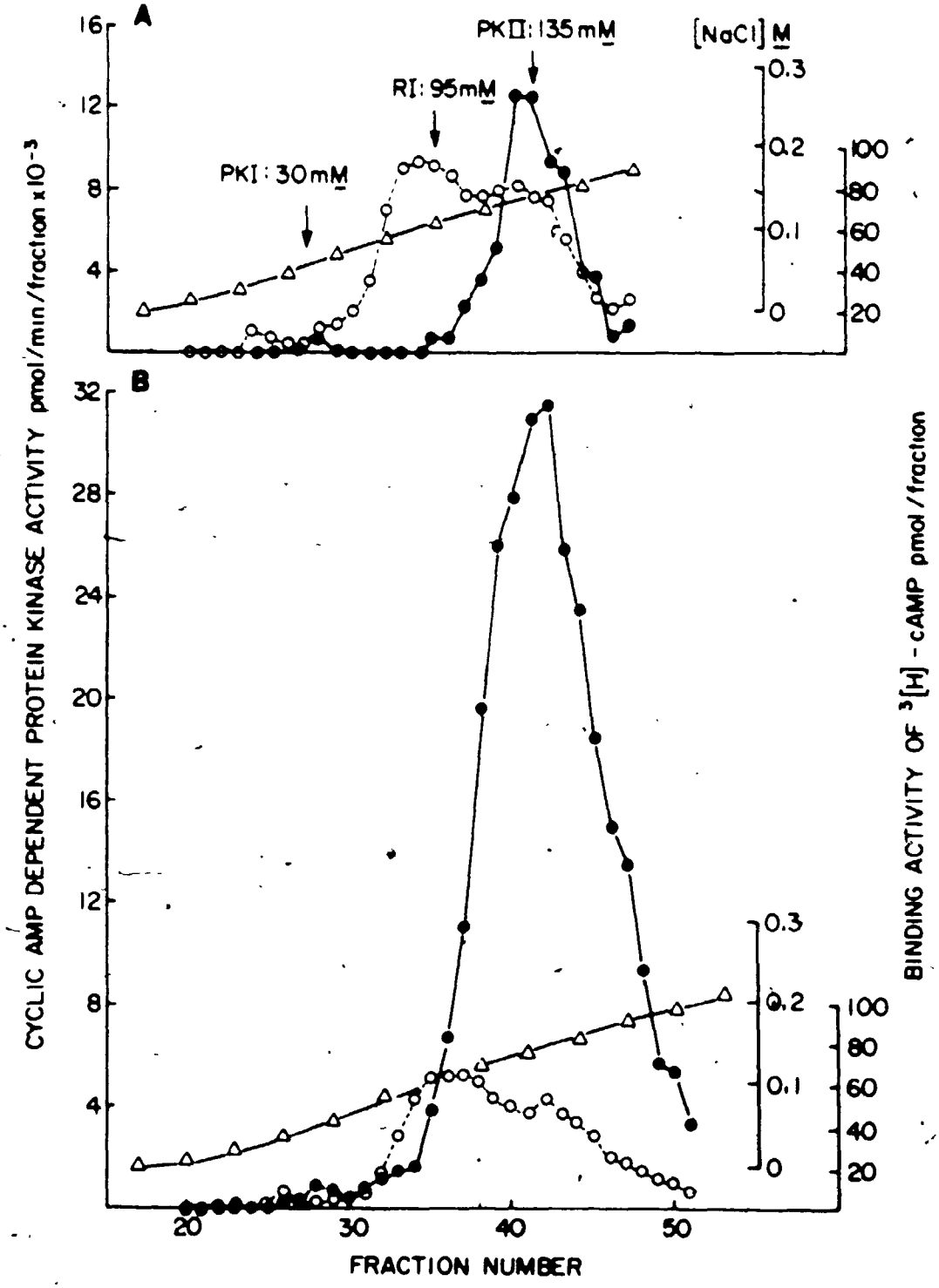
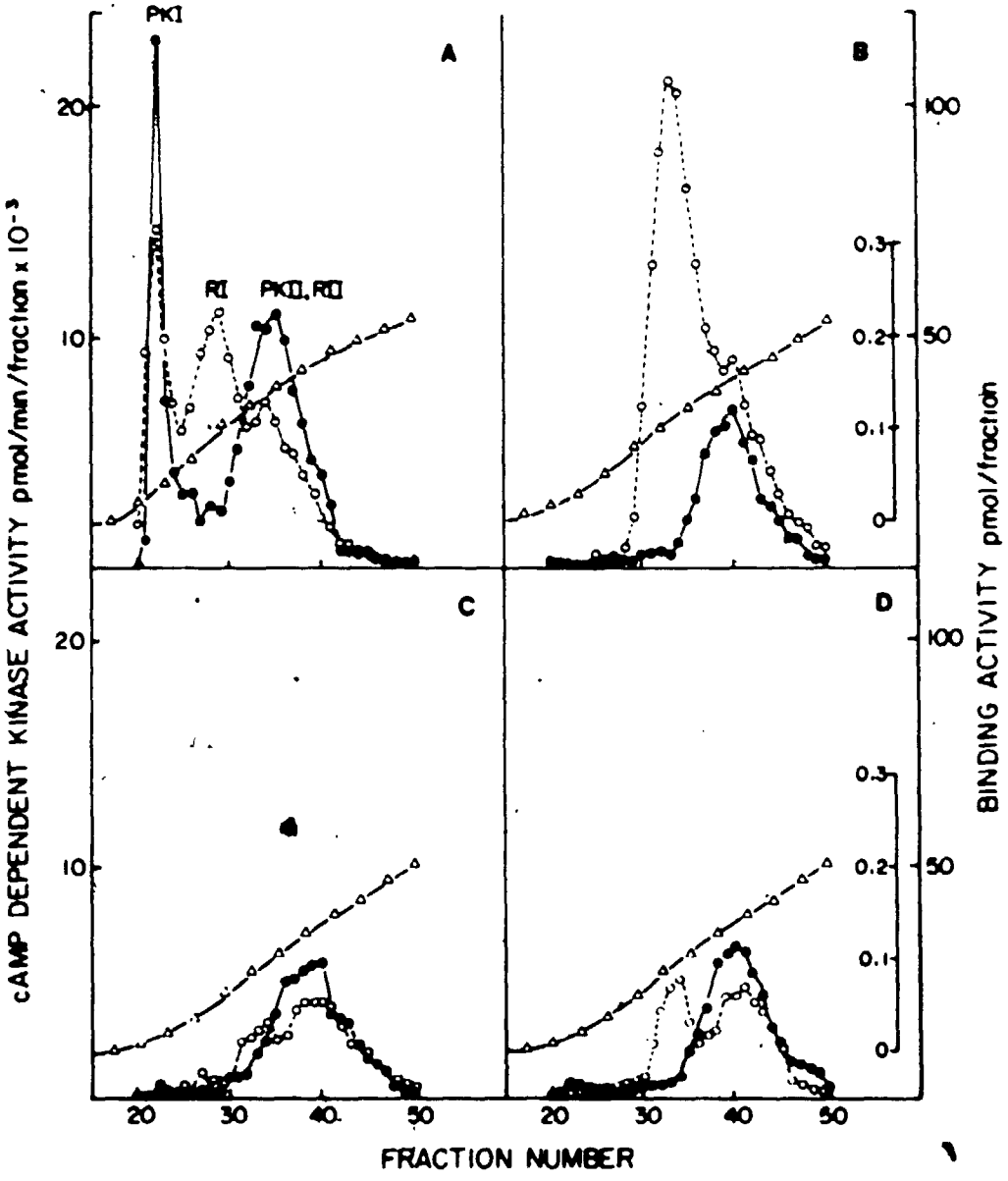

A
B
PKII: 135 mM
RI: 95 mM
PKI: 30 mM
[NaCl] M
0.3
0.2
0.1
0
100
80
60
40
20
16
12
8
4
32
28
24
20
16
12
8
4
20
30
40
50
FRACTION NUMBER
CYCLIC AMP DEPENDENT PROTEIN KINASE ACTIVITY pmol/min/fraction x10⁻³
BINDING ACTIVITY OF ³[H] - cAMP pmol / fraction

Figure 8 Chromatography of cAMP dependent protein kinases and their regulatory subunits in the cytosol fractions (40,000 xg) of L6 myoblasts. (A) untreated; (B) treated with 0.7 mM dbcAMP; (C) with 0.25 uM 8-Br cAMP; (D) with 10 μM forskolin. Assays for cAMP dependent protein kinase activity (●) and the regulatory subunits (○) were carried out as described in Figure 1. RI and RII were detected by binding to [^{3}H] cAMP. The NaCl elution gradient (△) was as in Figure 1. The positions of the peak fractions of PKI, RI, PKII and RII are indicated in panel A. All activities were normalized for 100 mg protein loaded. This figure reproduced with kind permission from Dr. S. Narindrasorasak from Beushausen et al. (1986).


PKI
A
B
C
D
RI
PKII, RII
20
10
100
50
0.3
0.2
0.1
0
20
30
40
50
cAMP DEPENDENT KINASE ACTIVITY pmol/min/fraction x 10⁻³
BINDING ACTIVITY pmol/fraction
FRACTION NUMBER

of PKI, a finding consistent with the reported absence of this enzyme from freshly isolated neural tissue (Weldon et al., 1985). The evidence presented here demonstrates that the predominant isozyme in primary cultures of rat astrocytes and oligodendrocytes is PKII (Figure 7), which is the enzyme species most abundant in mammalian CNS tissue (Nairn et al., 1985). For comparison with neural cells PK activity was assayed in L6 myoblasts. The L6 line evidently contained considerable amounts of PKI as well as PKII, as shown in Figure 8A, in confirmation of previous data (Rogers et al., 1985). Following treatment with dbcAMP the activity of PKI in L6 myoblasts was diminished or eliminated completely but that of PKII was evidently not affected (Figure 8B). Similarly, there was no effect on PKII levels after a 48 hr. treatment of neural cell cultures with dbcAMP (unpublished data).

Concerning any possible connection between PK activities and CV replication, it is unlikely that virus expression is contingent upon the presence of PKI because MHV3 and JHMV were produced in both PKI^+ L6 cells and PKI^- JRU_5 mutant myoblasts as well as in the PKI^- neural cells (Table 10).

4.6 Effects of cAMP Concentration Upon the Regulatory Subunits of PKI and PKII

Bechtel et al. (1977) have shown that the catalytic (C) subunits of PKI and PKII from rabbit skeletal muscle are identical, but the regulatory (R) subunits are quite distinct with respect to their

Table 10

Effect of cAMP Analogues on Coronavirus Replication in Rat Myoblasts

Cell Type	Treatment	Virus	Days After Infection	
			1	3
	Control	JHMV	6.98	22.00
		MHV3	1.08	4.08
L6	1mM dbcAMP	JHMV	< 0.1	< 0.1
		MHV3	< 0.1	< 0.1
	1mM 8-Br cAMP	JHMV	< 0.1	< 0.1
		MHV3	< 0.1	< 0.1
	Control	JHMV	ND	8.88
JRU_5		MHV3	ND	16.80
	1mM dbcAMP	JHMV	ND	< 0.1
		MHV3	ND	< 0.1

Note: All titres x 10^2 PFU/ml

Cells infected at confluence

Values listed represent data obtained from a single experiment and corroborate the trend observed in two previous trials.

physiochemical properties. Consequently, it has been suggested that the enzyme specificity of PKs resides in the R component (Kondrashin, 1985).

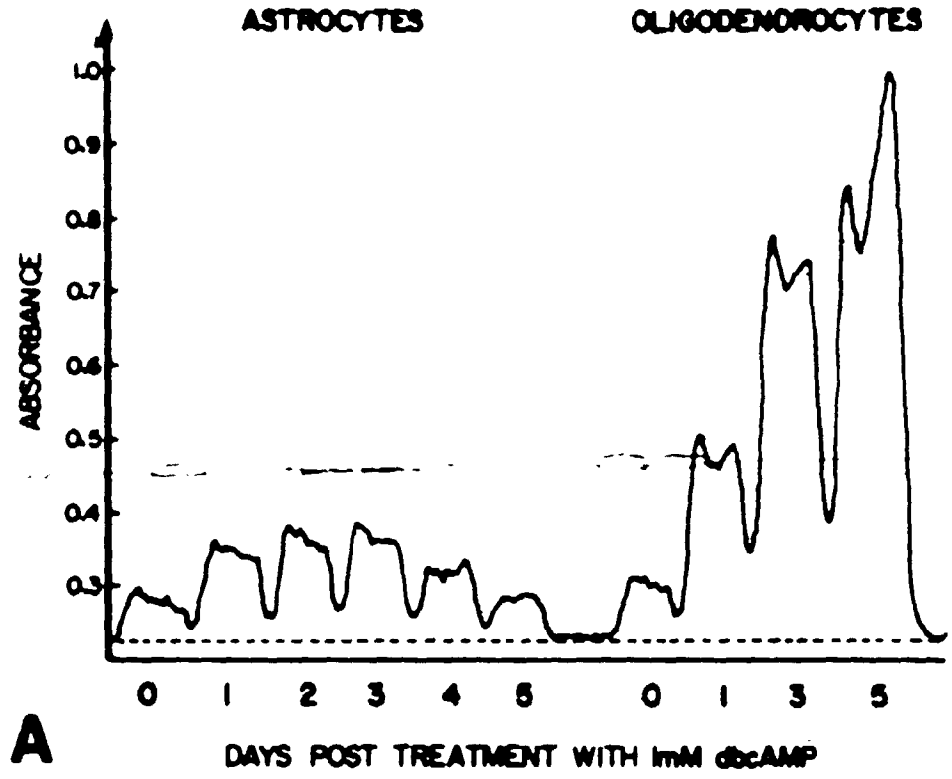
Changes in RI and RII of myoblasts, astrocytes and oligodendrocytes were monitored following treatment with inducers of differentiation. For this purpose either the binding of RI and RII to [^{3}H] cAMP or the covalent binding to the photoaffinity analogue 8-azido-[^{32}P] cAMP was determined. In the case of L6 myoblasts treatment for 40 hr. with 0.7 mM dbcAMP produced a dissociation of RI from the PKI holoenzyme, thereby generating more of the free form of RI. However, the net amount of RI was unchanged (Fig. 8A,B). The levels of the R subunits in astrocytes and oligodendrocytes were also monitored by photoaffinity labeling of cytosol extracts with 8-azido-[^{32}P] cAMP. It is clear from Fig. 9 that 8-azido-[^{32}P] cAMP binds specifically to polypeptides of MW 47,000 (47 K) and 52,000 (52 K), known from previous work to be RI and RII, respectively (Walter et al., 1979; Rogers et al., 1985). The binding analysis revealed that treatment of oligodendrocytes with 1 mM dbcAMP for 48 hr. caused a marked increase in the amount of RI despite the absence of PKI in these cells (Fig. 9). In contrast, the quantity of RII appeared to change very little.

The relationship between duration of treatment with 1 mM dbcAMP and the levels of RI and RII in astrocytes and oligodendrocytes was also assessed. The data, presented in Figure 10, show the relative amounts of RI as depicted in the densitometric tracing made from the original autoradiogram. Values derived by quantitating the area under

Figure 9 Photoaffinity labelling of RI and RII in the 100,000 x g cytosol extracts from primary rat oligodendrocytes. The autoradiogram of a 10% SDS-polyacrylamide gel demonstrates the specificity of 8-azido-[^{32}P] cAMP binding to RI and RII. Lanes 1 and 3 demonstrate the relative amounts of RI present in 5 ug of cytosol protein from cells not treated (lane 1) or exposed for 48 hours to 1 mM dbcAMP (lane 3). Lanes 2 and 4 are from identical samples to those in 1 and 3 respectively, except that a 50 fold excess of cAMP was included in the reaction.


RII
RI
4

Figure 10. Modulation of RI and RII in primary rat astrocytes and oligodendrocytes during treatment with 1mM dbcAMP. The concentrations of the regulatory subunits in cytosol (100,000 x g) fractions from astrocytes and oligodendrocytes were determined by binding of 8-azido-[^{32}P] cAMP, as described in Materials and Methods. In (A) a densitometric tracing made from the autoradiogram in (B), obtained after 10% SDS-polyacrylamide gel electrophoresis enabled a comparison of the time related changes in the regulatory subunits. Absorbance units have been normalized to the band of greatest density, (oligodendrocytes, 5 days post treatment).


ASTROCYTES
OLIGODENDROCYTES
ABSORBANCE
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0 1 2 3 4 5 0 1 3 5
DAYS POST TREATMENT WITH 1mM dbcAMP

A


RI

B

each peak indicated that astrocytes treated for 3 days increased 2.4 fold in RI but upon extension of the treatment to 5 days the amount of RI declined to the same level as that in untreated controls (Table 11). With RII, dbcAMP caused a maximal increase after 3 days to a level only 1.2 fold greater than that present in the untreated controls. However, the fifth day of treatment the RII concentration declined to 0.6 that of the controls.

In the case of oligodendrocytes, dbcAMP caused a rapid increase in free RI, so that within 24 hr. this component was about 3.5 times more abundant (Fig. 10, Table 11) and by the fifth day RI was increased almost 10 fold over the amount present in control cells. By comparison, the level of RII was increased very slowly to a value by the third day post treatment of 1.5 greater than in the controls. RII remained at this level until the fifth and final day of sampling.

As a test of the specificity of dbcAMP, treatment with 1 mM dbcGMP for 48 hr did not cause either an increase or decrease in RI of oligodendrocytes (Figure 11), consistent with the inability of this metabolite to inhibit CV replication (Table 8).

Exposure of myoblasts and neural cells to 8-Br cAMP, which also induces cell differentiation, suppressed CV replication in oligodendrocytes and L6 cells (Tables 7, 10). This result prompted an examination of the effect of 8-Br cAMP on concentrations of the RI subunit. It is evident from the data presented in Fig. 8, that treatment of L6 myoblasts with 1 mM 8-Br cAMP caused a decrease of RI as compared with the controls (Fig. 8A vs C). When oligodendrocytes were exposed to 8-Br cAMP the levels of RI, monitored by binding to

Table II

Modulation of Regulatory Subunits Upon Prolonged Exposure to dbcAMP in Primary Rat Neural Cells

Cell Type	Subunit	Days Post Treatment					
		0	1	2	3	4	5
Astrocytes	RI	1.00	2.01	2.10	2.43	1.52	1.00
	RII	1.00	1.00	1.02	1.21	0.82	0.61
Oligodendrocytes	RI	1.00	3.52	ND	7.34	ND	9.97
	RII	1.00	1.28	ND	1.48	ND	1.48

All values normalized to Day 0 of each row.

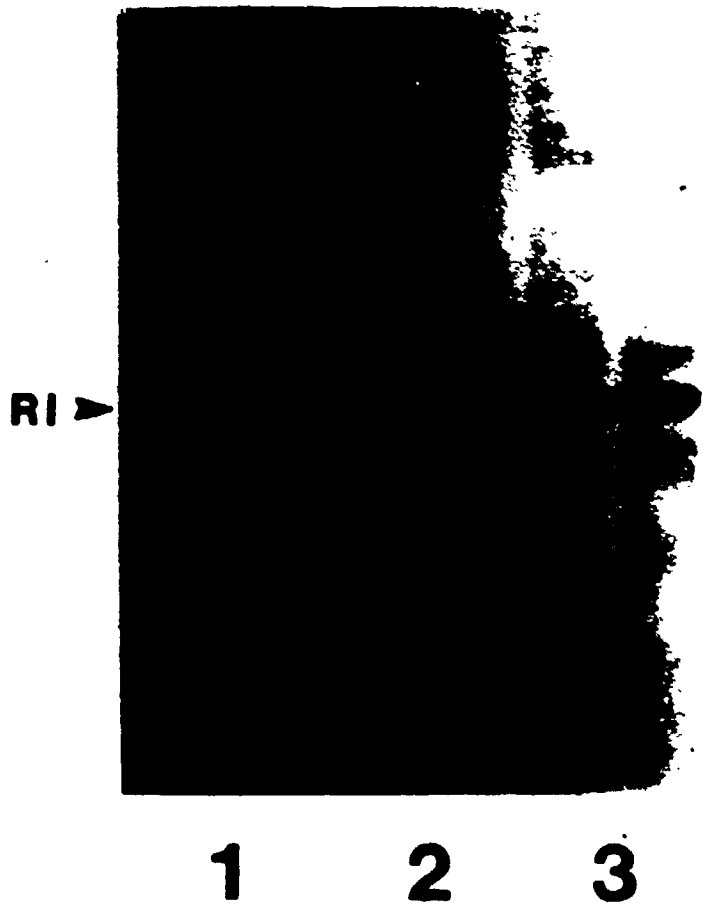
Figure 11 Modulation of regulatory subunits in primary rat oligodendrocytes following 48 hr exposure to BME_{10} (lane 1); 1 mM 8-Br cAMP (lane 2); 1 mM dbcAMP (lane 3) and 1 mM dbcGMP (lane 4). Cytosol (100,000 x g) extracts containing 5 ug protein were reacted with 8-azido-[^{32}P] cAMP and analyzed on 7.5-15% gradient SDS-polyacrylamide gel, as described in Materials and Methods.

1 2 3 4

8-azido-[^{32}P] cAMP, were also depressed as illustrated in Fig. 11. The reduction in amounts of RI in L6 myoblasts may be due to a conformational change produced by the binding of the bromine analogue to the RI molecule, which may cause a destabilization and increased turnover of RI (Ian Lorimer, private communication). To determine whether 8-Br cAMP did, indeed, accelerate turnover of RI in oligodendrocytes, cultures of these cells were exposed to dbcAMP for 48 hr to elevate RI levels, then were treated for 4 hr. with 1 mM 8-Br cAMP. The quantities of RI were determined both by Western blotting, for which Rabbit anti-Rat RI antiserum was used (data not shown) and by photoaffinity labelling with 8-azido-[^{32}P] cAMP, as described above. The binding of RI to 8-azido-[^{32}P], illustrated in Figure 12, indicated that less RI was present in cells treated briefly with 8-Br cAMP. Quantitative measurements by densitometry from the autoradiograms derived from both experiments showed that the 8-Bromo derivative reduced the amount of RI by about 24% of the controls treated only with dbcAMP. This result indicates that considerable loss of RI occurred during the 4 hr. treatment in the presence of 8-Br cAMP.

Treatment of L6 myoblasts with forskolin also caused a decrease in RI, as shown in Figure 8D. This effect may have been due to the interaction of forskolin either with the catalytic subunit of PKI or with the catalytic-G subunit complex (Barber and Goka, 1985), resulting in a dissociation of RI from the catalytic subunit by stimulating the adenylate cyclase system without interacting directly with RI. It is conceivable that RI was decreased because the cAMP

Figure 12 Turnover of RI induced with dbcAMP in primary rat oligodendrocytes during short exposure to 8-Br cAMP. Following a 48 hr treatment with 1 mM dbcAMP the cultures were exposed to 1 mM 8-Br cAMP and prepared for affinity labelling with 8-azido-[^{32}P] cAMP, as described in Materials and Methods. Untreated controls in duplicate are illustrated in 1; duplicate samples from cells treated with 1 mM dbcAMP for 48 hours in 2; duplicate samples from cells treated as in 2, then with 1 mM 8-Br cAMP for 4 hours, in 3.


RI
1
2
3

generated preferentially occupied site 1 on RI (Hoppe, 1985), which may have produced a conformational change in RI resulting in instability and accelerated turnover of this protein (Steinberg and Agard, 1981).

These observations support the idea that inhibition of CV replication in oligodendrocytes and L6 myoblasts is affected through the activation of the adenylate cyclase system manifested in the modulation of free RI, whether it be by an increase or more rapid turnover of the molecule.

4.7 Early Events in Host-Virus Interactions Following Treatment with Inducers of Differentiation

Several steps in early virus-cell interactions were examined, including adsorption, penetration and primary translation and transcription, to elucidate how JHMV replication might be blocked in terminally differentiated oligodendrocytes. It is evident from Table 12 that adsorption of JHMV to either astrocytes or oligodendrocytes was unaffected by prior exposure to 1 mM dbcAMP. It should be noted that ability of JHMV to become adsorbed onto astrocytes revealed that specificities in tropism, whereby JHMV fails to replicate in rat astrocytes (Beushausen and Dales, 1985), are not related to the absence of the relevant receptors. Although adsorption frequencies between the cell types varied such that approximately 30% less JHMV became adsorbed to astrocytes, there was no significant difference between proportion of cells to which virus became complexed, whether they originated from dbcAMP treated or untreated cultures.

Table 12

Adsorption of JHMV to Various Cell Types of Rat Origin

Cell Type	PFU	Cell Density	Frequency (Plaques/Cell)
Control Oligodendrocytes	2.64×10^4	2×10^6	1.32×10^{-2}
+ 1mM dbcAMP	2.00×10^4	2×10^6	1.0×10^{-2}
Control Astrocytes	2.80×10^3	8×10^5	3.6×10^{-3}
+ 1mM dbcAMP	2.80×10^3	8×10^5	3.5×10^{-3}
Control L6 Myoblasts	1.52×10^4	1.2×10^6	1.27×10^{-2}
+ 1mM dbcAMP	9.74×10^3	1.2×10^6	8.12×10^{-3}

Similarly, data on the rates of JHMV internalization (as defined in Materials and Methods) shown in Figure 13, demonstrated that the capacity by oligodendrocytes to take up the virus was unimpaired, regardless of prior treatment with 1 mM dbcAMP. These results showed that virus penetration reached a maximum of 10^3 PFU/culture at approximately 15 min after initiation of uptake by warming, then gradually fell during the subsequent 2 hrs. Evidently only a fraction of the inoculum became eclipsed, presumably due to loss of virion integrity.

To ascertain whether viral transcription and translation occurred in dbcAMP-treated oligodendrocytes, infected cultures were analyzed for JHMV RNA synthesis by means of dot blots and for antigens using antibodies employing the indirect immunofluoresence technique. It is evident from the dot blot data, presented in Figure 14, that JHMV-specific RNA could be isolated from productively infected oligodendrocytes but not from oligodendrocytes pretreated with 1 mM dbcAMP. The intensity of the signal provided by the [^{32}P] labelled cDNA probe was more sensitive for detection of JHMV RNA, than by [^{35}S] labelled probe as illustrated in Fig 14.

Failure to detect virus-specific antigens in primary oligodendrocytes pretreated with differentiation inducers and challenged with JHM, using M>MHV, support earlier claims that CV translation products were absent in differentiated cells.

CO;

Figure 13 Time course of JHMV association with primary rat oligodendrocytes. Titres of internalized JHMV recovered at various times after adsorption at 4°C for 60 min. and removal of extracellular inoculum with proteinase K from untreated oligodendrocytes (○) or oligodendrocytes pretreated for 48 h with 1 mM dbcAMP (△).

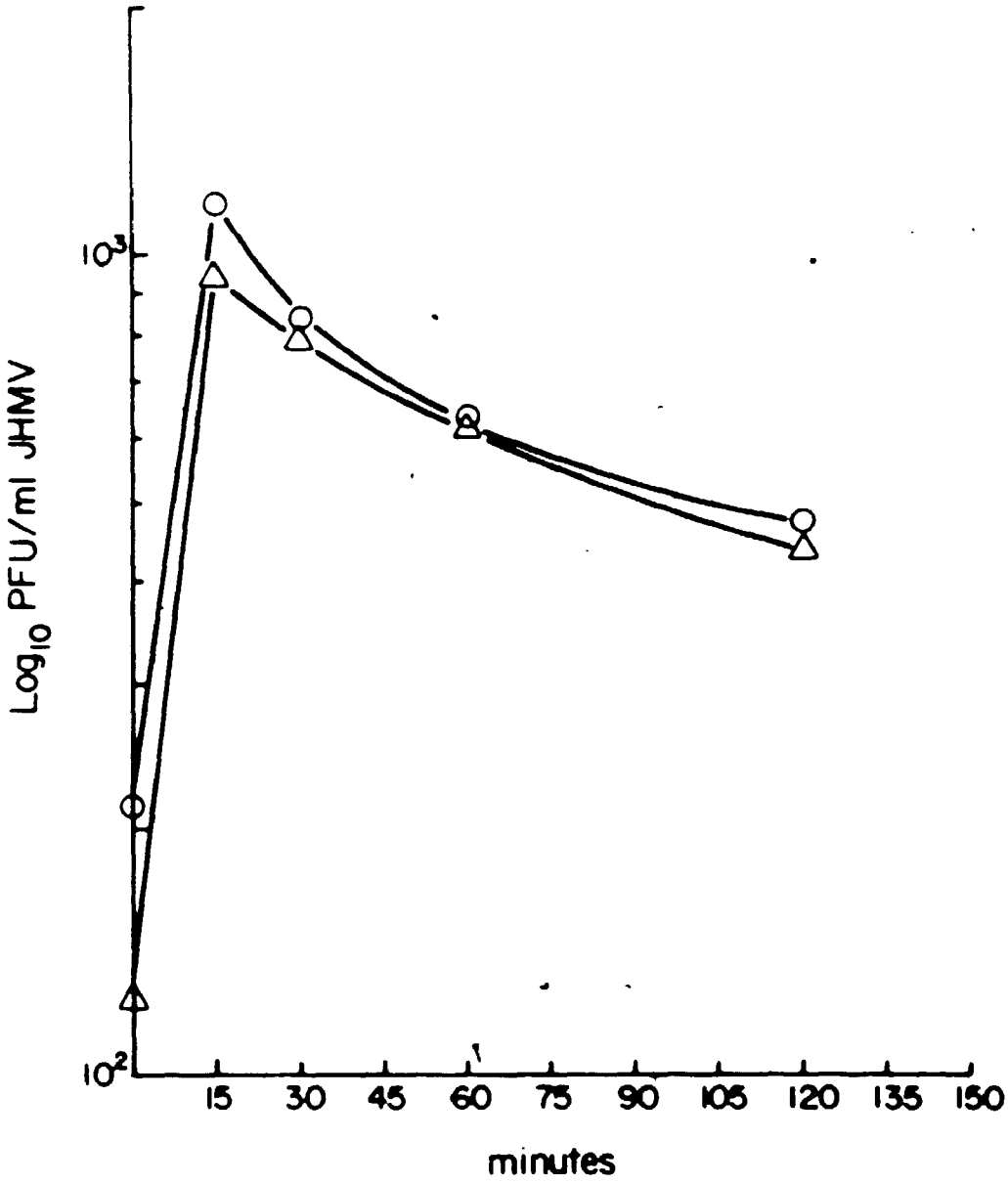
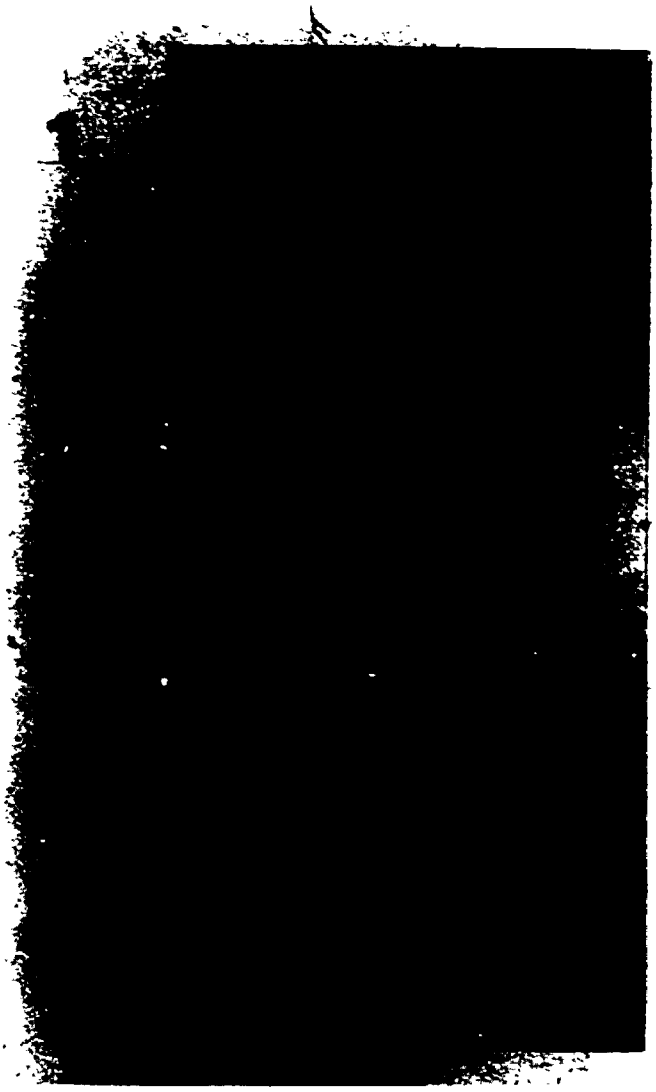

Log10 PFU/ml JHMV
10³
10²
15
30
45
60
75
90
105
120
135
150
minutes

Figure 14 Dot-blot analysis of cells infected with JHMV with [^{35}S] labeled cDNA to JHMV nucleocapsid RNA. Each row consists of RNA extracts blotted in a series of 1:9 dilutions from left to right, the left most dot containing undiluted extract. The samples include extracts from: 1. JHMV infected L-2 cells sampled 8 hr. post infection (p.i.); 2. Uninfected L-2 cells; 3. JHMV infected oligodendrocytes sampled 5 d.p.i., producing 10^3 PFU/ml; 4. JHMV infected oligodendrocytes, pretreated for 48 hr. with 1 mM dbcAMP, sampled 5 d.p.i.; 5. Poly A selected JHMV RNA isolated from infected L-2 cells; 6. JHMV infected oligodendrocytes sampled 5 d.p.i., producing 6 x 10^1 PFU/ml and probed with ^{32}P labelled cDNA to JHMV.



Since the block of JHMV replication in differentiated oligodendrocytes was pinpointed at a stage following penetration, the evidence indicated that the inhibition observed may somehow be connected with the virus uncoating process.

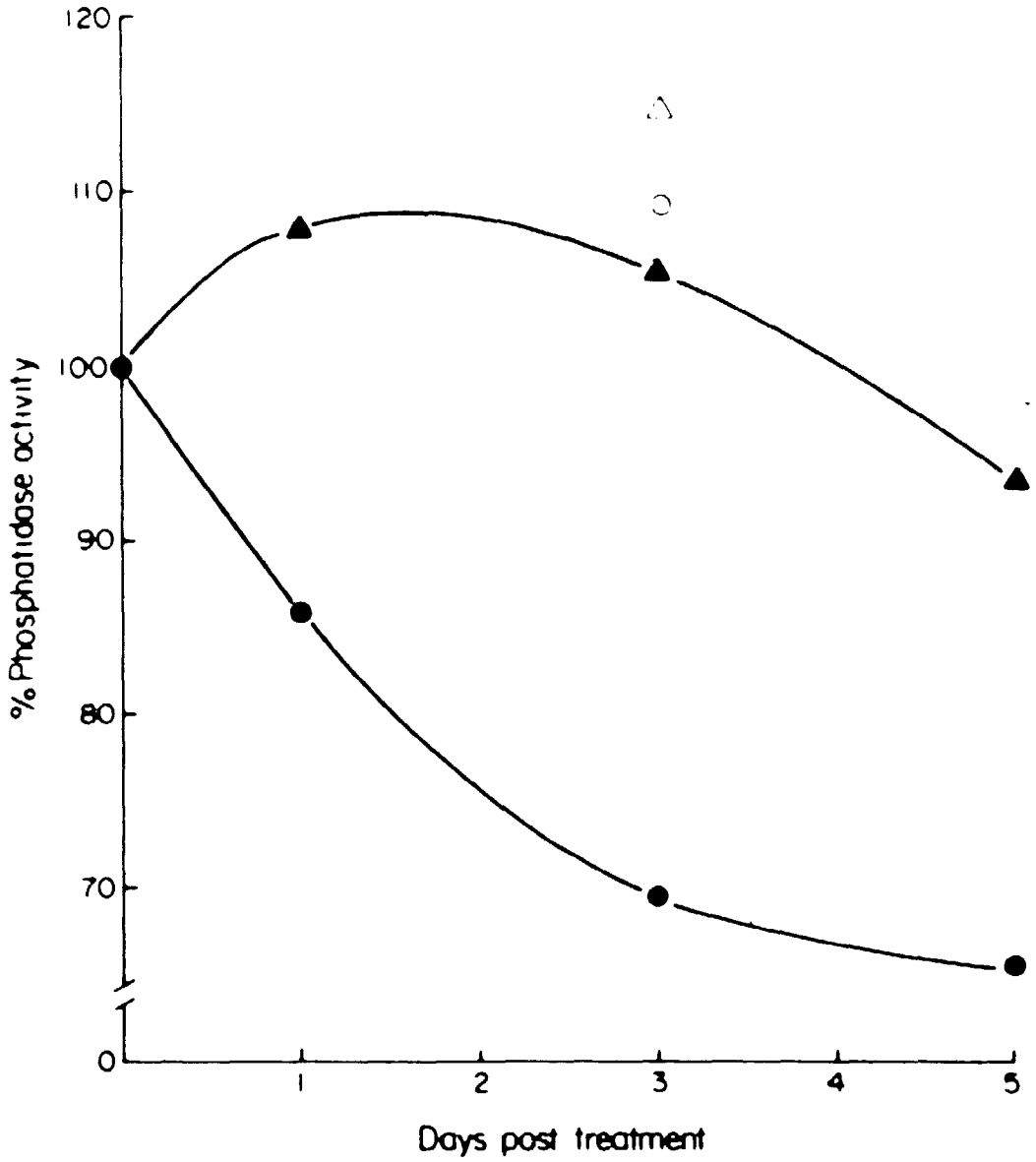
4.8 Effect of Differentiation on Acid Phosphatase Activities in Oligodendrocytes

Although the mechanism of CV uncoating has not been elucidated, the fact that in this group the plus stranded RNA genome is, by itself, infectious (Wege et al., 1978) implies that viral functions may commence after the genome has been divested of the NC protein coat, in which it is wrapped when inside the virus envelope. The presence of phosphorylated residues in the NC (Stohlman and Lai, 1979) suggested the possibility that uncoating of the RNA by removal of the NC from the genome is facilitated by one or more among the repetoire of host enzymes related to the cAMP-dependent protein kinase cascades. Therefore, repression of these enzymes in differentiated oligodendrocytes might be the underlying cause of arrested JHMV expression. This notion is consistent with the previous results which demonstrated existence of a relationship between the induction of cAMP-dependent protein kinase cascades, manifested by modulation of the levels of RI and the inhibition of JHMV expression. It is also in accord with the view of Ingebritsen and Cohen (1983) that protein phosphatases and kinases are important targets for cellular regulation. Based on this background information and evidence for CV penetration via endosomes (Krzystyniak and Dupuy, 1984), organelles

which possess very high activities of acid phosphatase (De Duve et al., 1955; Paigen and Griffiths, 1958), the relationship between differentiation and acid phosphatases of oligodendrocytes was examined.

The initial assays were carried out with soluble and particulate extracts of oligodendrocytes, in the presence of the divalent cation Mn^{++} and para-nitrophenyl phosphate (pNPP) as the substrate. Material in the particulate fraction from oligodendrocyte cultures treated with 1 mM dbcAMP possessed lower acid phosphatase activities than material from the controls. There was a relationship between the amount of inhibition observed and the duration of treatment, as shown in Figure 15. By contrast, acid phosphatase activities in the supernatant fraction were relatively unaffected, regardless of the duration of treatment. Application of 8-Br cAMP for 3 days, so as to induce oligodendrocyte differentiation, did not reduce the particulate acid phosphatase activities but may actually have elevated them slightly over that of controls (Fig 15). An inverse correlation appeared to exist between levels of acid phosphatase activities in the particulate fractions and the quantity of RI which accumulated following treatment with inducers of differentiation, shown in Figure 10. It should, however, be understood that information from assays on non-physiologic substrates such as pNPP, when employed to identify and measure specific protein phosphatase activities, is of limited value (for review see Sparks and Brautigan, 1986). Therefore, it became essential to determine optimum conditions for the phosphatases presumed to dephosphorylate the NC protein of CV.

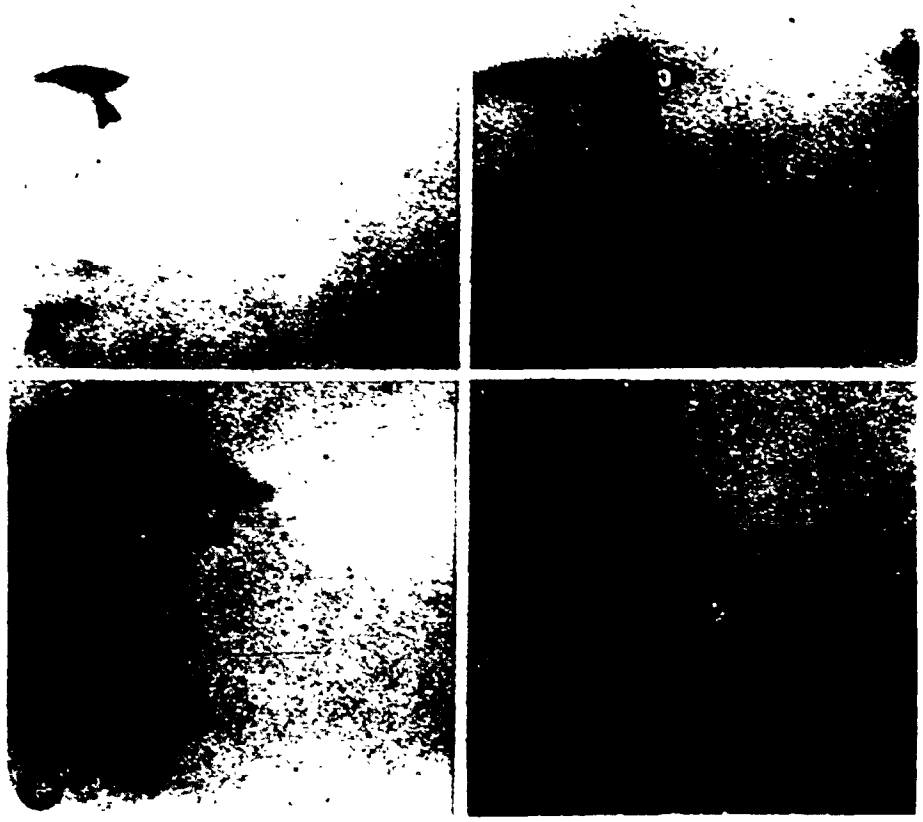
Figure 15 Acid phosphatase activity in primary oligodendrocytes. Replicate cultures of oligodendrocytes treated with 1 mM dbcAMP (solid) or 1mM 8-Br cAMP (open) were separated into the supernatant (▲) and 100,000 xg particulate fractions (●) and assayed for acid phosphatase activity using pNPP as the substrate. Following 30 min. incubation at 30°C the reaction product was measured at 410 nm. The data are expressed as percentage of activity in untreated cells sampled on the 5th day.


120
110
100
90
80
70
0
% Phosphatidase activity
1
2
3
4
5
Days post treatment

It should be recalled that eukaryotic protein phosphatases belong to 2 major groups, one specific for phosphoserine or phosphothreonine residues and the other acting on phosphotyrosines (Sparks and Brautigan, 1986). Additional parameters which may influence optimal activity include the ionic strength of the reaction mixture, the pH, temperature and divalent cation requirements.

In this study, acid phosphatase(s) in the particulate fraction of oligodendrocytes were tested for their ability to dephosphorylate isotopically labelled NC, shown previously to be phosphorylated exclusively on serine residues (Stohlman and Lai, 1979). After mixing with cell extracts the [^{35}S]-NC protein underwent processing, as evident from Figure 16. When Fe^{++} was present in the reaction mixture some of the p56 NC recovered by electroelution from SDS-PAGE gels was modified, perhaps due to dephosphorylation, as indicated in Fig 16B by the changed position in the gels, usually ascribed to charge differences. The [^{35}S] NC protein isolated in cell extracts without denaturation, when used in the reaction with particulate material from oligodendrocyte cultures, underwent processing even further and demonstrated molecular weight shifts in part to intermediates p50 and P40, as illustrated in Figures 16C vs D. The products arising from this reaction appear in the 2D gels immediately to the right of and slightly below the viral protein. This pattern of precursor processing is also evident from pulse-chase experiments and 1-D SDS-PAGE analyses of [^{35}S] labelled extracts of JHMV infected L-2 cells

Figure 16 Processing of JHMV NC protein under conditions favourable for Fe^{++} dependent acid phosphatase activity. In A and B 100 ug of protein from 100,000 x g oligodendrocyte particulate material was incubated for 90 min. at 30°C (pH 5.5) in the absence (A) or presence (B) of 1 mM Fe^{++} and analyzed using the 2-dimensional gel system of O'Farrell (1975). Arrowheads indicate the positions of NC. Note in B the additional spot, presumably arising from a change in charge. To emphasize the spots the autoradiogram in B was exposed for 20 days while that in A for 5 days. In C and D the material used in the reaction, carried out as above, was [^{35}S] labelled JHMV NC recovered from infected L-2 cells and analyzed using the 2-D gel system of Mets and Bogorad (1974). In C, the reaction was carried out without Fe^{++}; in D processing to p40 was evident in the presence of Fe^{++}.



shown in Figure 20C. It is quite possible that the p40 product may have been derived by proteolytic cleavage in conjunction with or soon after dephosphorylation of the p56 NC.

To demonstrate more directly a precursor-product relationship between p56 and p40, the two polypeptides were subjected to 2-dimensional tryptic peptide mapping. As evident from Figure 17, the [^{32}P] labelled peptide fragments of p40 constitute a subset of those generated from p56 regardless of the chromatographic solvent system used in the analysis (Fig 17A,B vs C,D). There was an absence of 2 prominent ^{32}P-labelled spots from the peptide map of p40 which may be due either to dephosphorylation or loss of the relevant region on p56 after proteolytic cleavage or as the consequence of both events.

The relationship between treatment of oligodendrocytes with differentiation inducers and activity of NC-related phosphatase(s) in the particulate fraction was also examined. It appears from the data in Figure 18 that extracts derived from untreated control cultures produced more efficient processing of the [^{32}P] NC protein than did comparable extracts from cultures treated with either dbcAMP or 8-Br cAMP as judged by the quantity of p40 appearing. These observations are not inconsistent with the hypothesis that acid phosphatase(s) function to dephosphorylate the NC during normal CV penetration and uncoating, a process which is repressed in oligodendrocytes driven towards differentiation.

As a further proof of substrate specificity for the NC by Fe^{++} dependent acidic phosphatase(s), extracts from the oligodendrocyte particulate fraction, obtained from control cultures or those treated

Figure 17 Two dimensional phosphotryptic peptide analysis of JHMV NC protein, p56, (A,C) and the p40 cleavage product (B,D). Chromatography in two separate solvent systems (A, B vs C, D) demonstrated that p40 is related to p56. Large and small arrowheads indicate the absence of major and minor phosphopeptides, respectively.

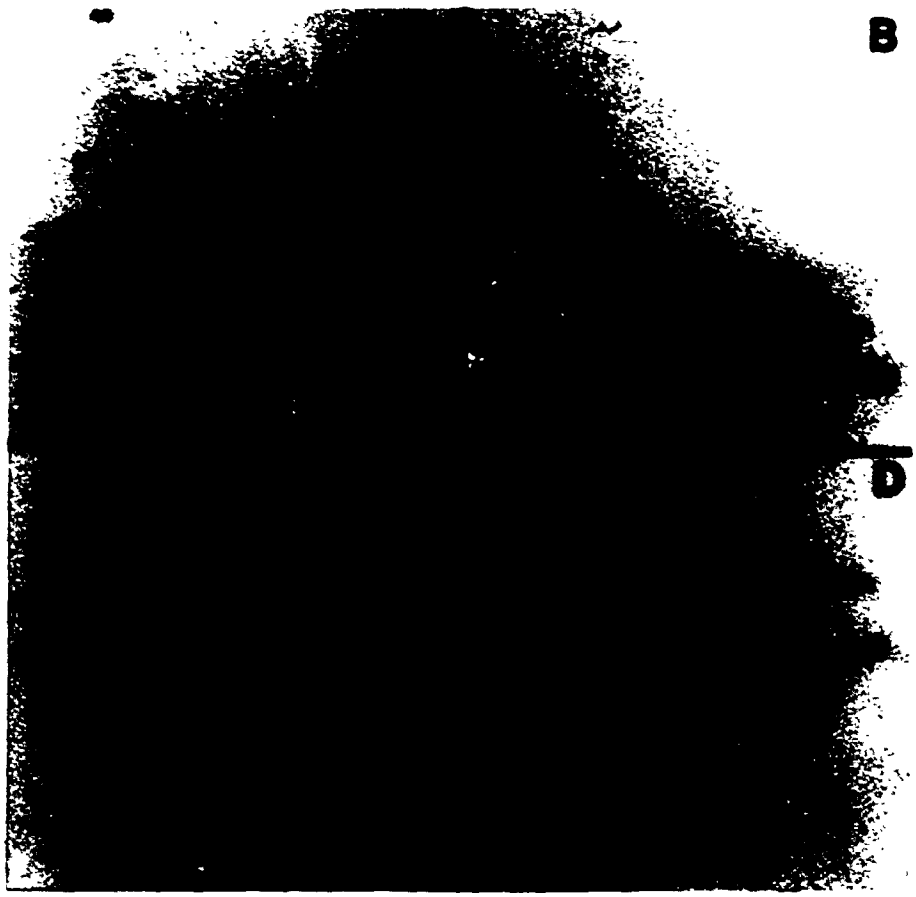
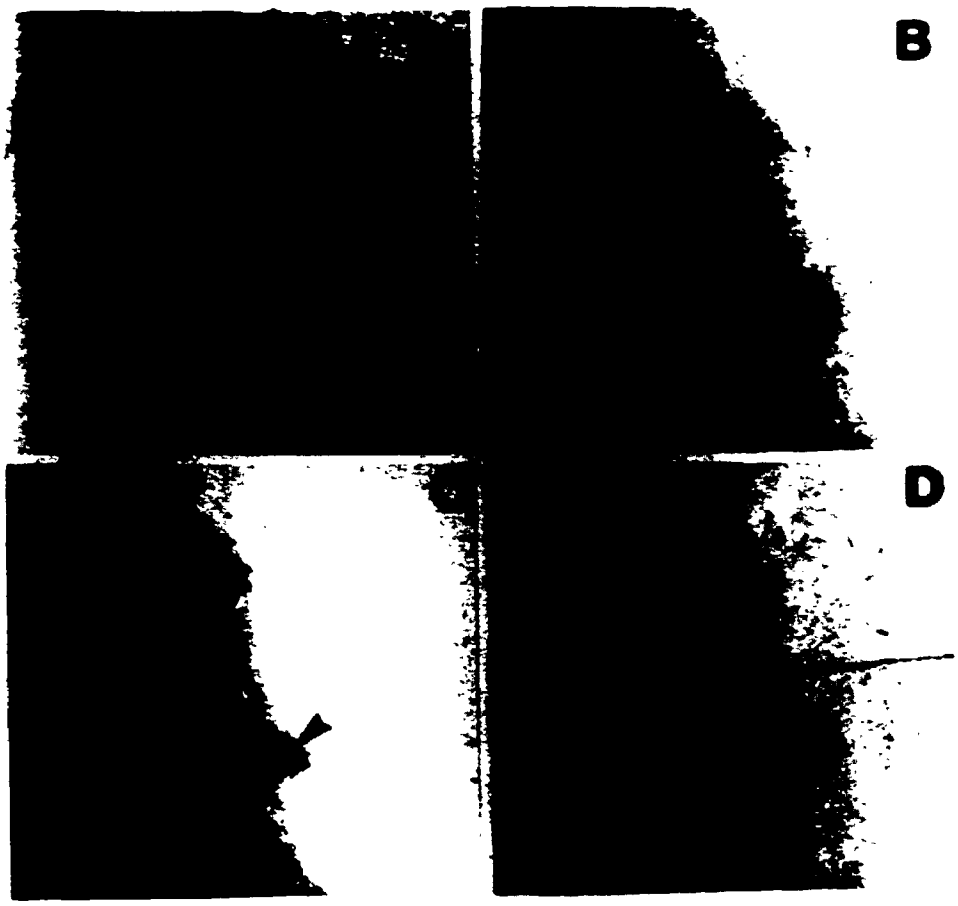

B
D

Figure 18 Two dimensional gel analysis of processing of [^{32}P] labelled JHMV NC using conditions favouring Fe^{++} dependent acid phosphatase activity. NC material was incubated with 50 ug protein from 100,000 x g oligodendrocyte particulate fractions for 60 min. at 30°C and analyzed by the 2D gel system of Mets and Bogorad (1974). In A and C particulate material from untreated oligodendrocytes incubated in the presence or absence of 1 mM Fe^{++}; B, D particulate material from oligodendrocytes pretreated with 1 mM dbcAMP (B) or 1 mM 8-Br cAMP (D), incubated in the presence of 1 mM Fe^{++}. Arrowheads indicate the position of p40.

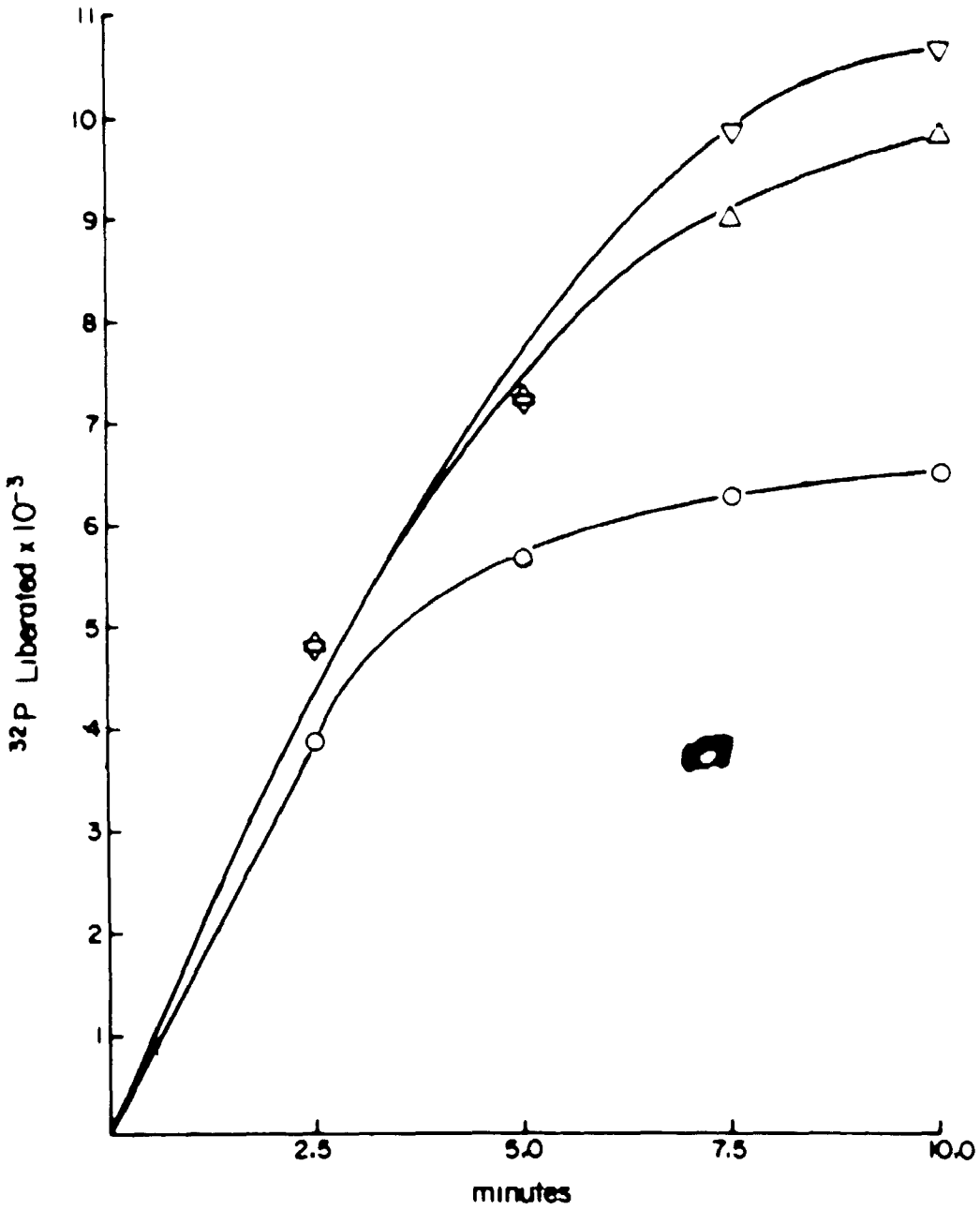

B
D

with dbcAMP or 8-Br cAMP, were tested on [^{32}P]-labelled α-casein as the substrate. Data on the release of [^{32}P] during incubation with cell extracts, shown in Figure 19, revealed that after differentiation dephosphorylating activity against α-casein was increased, contrary to the situation with NC. This demonstrates that caution should be exercised in interpreting results from experiments using non-specific substrates for demonstrating specificity of a phosphatase activity when the cell contains a large spectrum of enzymes possessing broad specificities.

4.9 Affinity for Binding Nucleic Acid by the Nucleocapsid Protein and its Products

Robbins et al (1986) have recently shown by means of a sensitive nucleic acid binding overlay-protein blot assay that p56 of CV A59 is a nucleic acid binding protein and suggested existence of a relationship between phosphorylation of the NC and a capacity to bind the viral genome. This suggestion fits the hypothesis regarding the uncoating of CV, which may involve dephosphorylation of the p56 NC, followed by proteolysis so as to facilitate release of NC to yield free genomic RNA. For this reason the capacity of p56 and products p50 and p40 for binding JHMV RNA labelled with [^{32}P] and JHMV cDNA labelled with [^{35}S] was tested. The polypeptides with potential for nucleic acid binding were resolved in 1-D SDS-PAGE. To identify the position of p56 and products, JHMV infected L-2 cells were subjected to labelling with [^{35}S] methionine in pulse chase experiments. The SDS-PAGE analysis of extracts, illustrated in Figure 20C showed that

Figure 19 Dephosphorylation of [^{32}P] labelled ∝-casein, employing conditions favouring Fe^{++} dependent acid phosphatase activity. Phosphorylated ∝-casein was incubated with 50 ug of 100,000 x g particulate material from untreated oligodendrocytes (○) or oligodendrocytes pretreated with 1 mM dbcAMP (△) or 1 mM 8-Br cAMP (▽). Reactions run at 30°C were terminated at 2.5 min. intervals and the liberated [^{32}P] phosphorous extracted and assayed according to Maeno and Greengard, (1972).

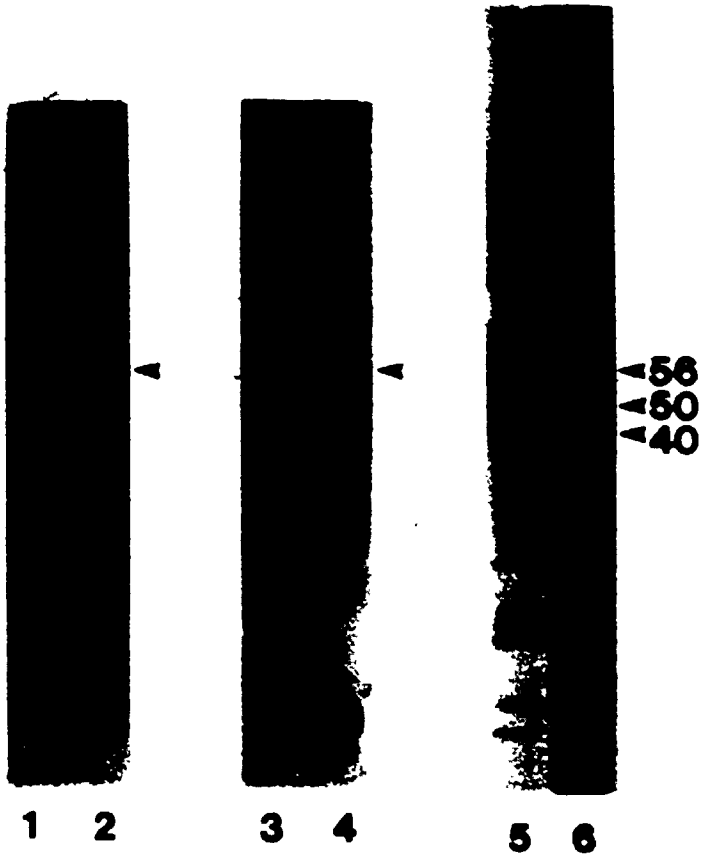

^{32}P Liberated x 10^{-3}
11
10
9
8
7
6
5
4
3
2
1
2.5
5.0
7.5
10.0
minutes

NC p56 was partially converted into p50, a related protein indistinguishable from p56 by [^{35}S] 2-D tryptic peptide mapping (Cheley and Anderson, 1981; Sidell, 1982) and also into p40, shown in Figure 17 above to be a processed product of NC.

It is evident from Figure 20 that p56 was the prominent JHMV protein binding nucleic acid, confirming the findings of Robbins et al (1986). It may be highly significant that neither the RNA or cDNA probe was adsorbed to the polypeptide band at the position of p40, indicating that this component lacked the relevant domain for binding nucleic acid. This observation is consistent with the hypothesis that uncoating involves a host cell-controlled separation of NC from the viral genome, permitting the free RNA to initiate functions in the replication cycle.

Comment about the affinity of p50 for these nucleic acids must be reserved since any specificity which may have existed was obscured by the presence of a cellular protein comigrating with p50 that also bound the nucleic acid probes, as indicated by its presence in the uninfected cell extracts (Fig 20 lanes 1,3).

Figure 20 Nucleic acid overlay protein binding assay analysis demonstrating the affinity of NC and its products for nucleic acids. Binding of JHMV specific [^{35}S] cDNA (lanes 1 and 2) or [^{32}P] labelled JHMV RNA (lanes 3 and 4) to extracts from uninfected L-2 cells (lanes 1 and 3) and JHMV-infected L-2 cells (lanes 2 and 4). Arrowheads indicate the position of p56. The position of p56 is indicated in extracts from JHMV-infected L-2 cells pulsed with [^{35}S] methionine for 30 min. late in the infection (lane 5). Upon pulse labelling for 2 h. early in the asynchronous infection and chasing for 2 hr, both the p56 NC and its products p50 and p40 are prominent (lane 6). It should be noted that many host polypeptides were labelled due to incomplete shut down of host protein synthesis as a consequence of asynchronous infection at the early time point.


56
60
40
1 2
3 4
5 6

CHAPTER 5

DISCUSSION

5.1 Tropism and Differentiation Regulate the Infectious Process of Coronavirus in Primary Explants of the Rat CNS

The *in vitro* rat-coronavirus model under investigation here has provided significant new information concerning the infectious and disease process in the CNS. The closely parallel, age-related, inducibility of disease in animals and infectability of freshly explanted oligodendrocytes by the neurotropic agent JHMV is remarkable. This suggests that the *in vitro* tropism of this virus strain reflects accurately its propensity for cells of the CNS in the rat. Furthermore, the idea that JHMV has specific tropism for the oligodendrocytes is supported by data derived from an application of molecular probes and immunopathology to CNS samples from afflicted or asymptomatic animals (Sorensen, 1984). Therefore, it is very likely that progress of the chronic, demyelinating disease is related to infection of the oligodendrocyte. One might also presume that tropism of MHV_3 for astrocytes, causing a persistent *in vitro* infection, does not lead to development of an overt CNS disease in the rat because the infected astrocytes, are presumed to be cleared from the CNS and by virtue of their proliferative capacity are not eliminated from the CNS as critical, functional, components.

The uniqueness of differential tropisms of CV in the rat CNS appears to be species-specific, as indicated by comparison of results on rat and murine cells. In the latter, JHMV can replicate equally

well in astrocytes, neurons (Knobler et al., 1981a,b; Dubois-Dalcq et al., 1982; Collins et al., 1983) (Wilson et al., 1984) and oligodendrocytes (Wilson et al., 1986). The in vitro infectability of rat neurons remains to be demonstrated, although infection of these cells within the CNS has been documented (Nagashima et al., 1978; Sorensen et al., 1984). In future studies it should be possible to ascertain whether the CV serotypes infecting man are similarly characterized by cell-type related tropisms within the human CNS.

It should be noted that prompt and reproducible initiation of persistent infection by CV in primary cultures, described here, is very similar to the establishment of such infections in continuous cell lines of rat cells of neural and other origin (Lucas et al., 1977). Moreover, with both types of cultures production of infectious virus is profoundly inhibited or entirely suppressed at elevated temperatures, approximately 39.5°C. Analogous data have been obtained with other neurotropic agents, among them MV (Lucas et al., 1978) and Sendai virus strain HVJ (Ogura et al., 1984). Evidently, similar host cell control over virus production is exerted by primary neural cells, suggesting that the efficiency of virus replication and spread within the CNS may be under close control of the host. It is noteworthy that JHMV replication in explanted oligodendrocytes is influenced more profoundly by cell density than by the m.o.i. employed, suggesting that cell-cell contacts impart a fundamental influence upon the spread of virus and maintenance of persistence. Thus, in sparsely seeded cultures, where cell cohtacts are infrequent or absent, virus production is low and transient. By comparison, with greater density,

permitting frequent cell-cell contacts, the infection is characterized by development of syncytia and prolonged, persistent release of JHMV, sometimes to titres as much as four $\log_{10}$ greater than those produced by cells in sparse cultures.

A primary factor governing JHMV related demyelinating disease is the age at inoculation (Sorensen et al., 1980, 1982). Thus, 3 weeks post-partum, rats become highly resistant. With this in mind it should be noted that rat oligodendrocytes in vitro exhibit an age-related control over the infection, perhaps as a consequence of the differentiation process. In this connection, there is evidence to show that in neonatal and embryonic cultures from rat brain, certain characteristics of differentiation, such as the induction of MBP (Barbarese and Pfeiffer, 1981; Pfeiffer et al., 1981) and CNPase (Pfeiffer et al., 1981; McMorris, 1983), appear on schedule during development as regulated by the in vitro 'time clock' period. The consequence of differentiation on virus replication in neural cells, whether on the positive or negative side, has also been documented for other neurotropic agents, among them MV and rubella virus (Robbins and Rapp, 1980; Miller and Carrigan, 1982; Van Alstyne and Paty, 1983; Yoshikawa and Yamanouchi, 1984). It may not be a mere coincidence that the maximal increase in CNPase occurs both in vivo and in vitro at about 15-20 days post-partum (Sprinkle et al., 1978; McMorris, 1983), closely coincident with the time period at which rats become insusceptible to disease produced by JHMV; the key factor in regulation of infectability of oligodendrocytes could be their state of differentiation with respect to the elaboration of myelin. This

5.3 Mechanism of Inhibition of Coronavirus Replication in Differentiating Oligodendrocytes: Evidence for Inhibition of JHMV Nucleocapsid Uncoating

The mechanism through which differentiation inducers cause a repression of CV in oligodendrocytes, shown to be interrelated with modulation of the adenylate cyclase system, has been studied with emphasis on metabolic events related to the cAMP-dependent kinase cascades. Compounds such as dbcAMP and 8-Br cAMP, when applied prior to but not after inoculation, do not inhibit attachment or sequestration of the inoculum but do appear to prevent the expression of viral RNA and translation products. The evidence provided indicates that a block occurs at some stage after uptake but prior to expression of genomic functions, drawing attention to uncoating as the critical event.

The presumed function of cAMP as a "second messenger", mediating multiple effects through a cascade of activities connecting the cAMP-dependent protein kinases, has drawn attention to the possibility that viral functions are regulated in differentiating cells by kinases and/or phosphatases, two classes of enzymes which participate in the regulation of normal cellular functions (for review see Ingebritsen and Cohen, 1983). This idea may have considerable bearing on the mechanisms involved in the establishment of latent or persistent viral infections within the differentiating CNS. Phosphorylation of NC proteins of various RNA agents has been connected with different aspects of virus expression including the stimulation of influenza virus transcription (Kamata and Watanabe, 1977) and the regulation of

replication of CV. These observations may have a direct bearing on the age-related resistance to JHMV, which develops in preweanling rats (Sorensen et al., 1980) and oligodendrocyte cultures in vitro (Beushausen and Dales, 1985), at the time CNS myelination and oligodendrocyte differentiation are being completed.

In other cell-virus systems the same differentiation inducers as those used here can either activate or suppress infection, depending on the particular system being tested. For example, in the human amnion cell line, AV_3, treatment with cAMP following infection inhibits MV production (Robbins and Rapp, 1980). Likewise, compounds that stimulate differentiation by increasing intracellular levels of cAMP in neuronal cells, including papaverine, cAMP, dbcAMP, 8-Br cAMP and isobutylmethylxanthine repress the production of MV (Miller and Carrigan, 1982; Yoshikawa and Yamanouchi, 1984), emphasizing an apparent linkage between increased intracellular cAMP levels, differentiation and suppression of virus replication. Since VSV and MV were reported to replicate normally in primary rat oligodendrocytes pretreated with dbcAMP (Beushausen and Dales, 1985), the control over virus expression may be somewhat specific for each virus group and the particular host cell type involved. Similarities of the effects on CV expression produced by dbcAMP and other metabolites in L6 myoblasts and rat oligodendrocytes suggest that these effects on CV replication are due to changes in PK metabolism, more specifically to increases or accelerated turnover of free RI in the cytosol. Consistent with this viewpoint is the observation that CV replication is unimpeded when

there occur relatively small increases in RI following treatment of primary rat astrocytes with dbcAMP, reported here and by Loeffler et al. (1985), and L-2 fibroblasts (Table 13).

Recently, correlations have been made between the induction of differentiation and increased amounts of RI or RII, depending on the cell type under investigation (Prashad and Rosenberg, 1978; Walter et al., 1979; Schwartz and Rubin, 1983; Sato et al., 1985; Rogers et al., 1985; Loeffler et al., 1985). The evidence gathered here demonstrate for the first time that a relationship between differentiation, increases in free RI and inhibition of CV replication exists. It may appear contradictory from the preceeding statement that 8-Br cAMP and forskolin, which suppress CV replication, fail to bring about an increase in free RI in L6 myoblasts and primary rat oligodendrocytes. This anomaly can be explained if several facts regarding the interaction of cAMP with the regulatory subunits of the cAMP-dependent protein kinases are considered. First of all, it has been shown by Robinson-Steiner and Corbin (1982) that two cAMP binding sites, designated site 1 and site 2, exist on each regulatory subunit polypeptide chain. Analogues of cAMP substituted in the C8 position, such as 8-Br cAMP, bind more avidly to site 1 whereas those substituted in the N6 position, such as dbcAMP, have a predilection for site 2. Furthermore, it is known that the rate for cAMP exchange at site 2 is 10 fold greater than at site 1 (Flockhart and Corbin, 1982), making it highly probable that site 1 is the first to be fully occupied (Hoppe, 1985). The accelerated rate of turnover of RI is explicable on the basis of the suggestion that the binding of cAMP or

analogue to site 1 causes a conformational change in the regulatory subunit thus predisposing the molecule to proteolytic degradation (Steinberg and Agard, 1981). This idea makes it plausible that exposure of cells to either 8-Br cAMP or forskolin brings about an apparent decrease, rather than an increase, in the amount of RI due to its breakdown, despite the fact that treated cells are stimulated to differentiate.

Based on data from this study endeavors have been made to relate the metabolism of cAMP-dependent PK components with control over CV expression by combining the relevant observations presented in Table 13. From an inspection of this table a correlation between CV production and RI metabolism becomes evident, in terms of either an increase in free RI, as seen in dbcAMP treated oligodendrocytes and L6 myoblasts or decrease in amount, i.e. enhanced turnover of RI, caused by 8-Br cAMP and forskolin.

Although inhibition of CV replication associated with differentiation does not entirely account for the progress of the in vivo disease (Sorensen et al, 1986), it does present an attractive hypothesis for exploring the mechanisms controlling persistent and latent virus infections in the CNS.

Table 13

Relationship Between cAMP-Dependent Protein Kinases, Regulatory Subunits and Coronavirus Replication

Cell Type	Treatment	cAMP-Dependent Protein Kinases		Regulatory Subunits		Virus Replication	
		PKI	PKII	RI	RII	JHMV	MHV3
L2 Mouse	Control	+-	++	+	ND	++++	++++
Fibroblasts	1mM dbcAMP	-	++	+	ND	++++	++++
Rat	Control	-	++	+	++	-	+++
Astrocytes	1mM dbcAMP	-	++	++	++	-	+++
Rat	Control	-	++	+	++	+++	-
Oligodendrocytes	1mM dbcAMP	+-	++	+++++	++	-	-
L6 Rat	Control	+++	++	*	++	+	+
Myoblasts	1mM dbcAMP	-	++	**	++	-	-

*Increases with time within the cell

**Increase observed only in L6 subline JRU_5

5.3 Mechanism of Inhibition of Coronavirus Replication in Differentiating Oligodendrocytes: Evidence for Inhibition of JHMV Nucleocapsid Uncoating

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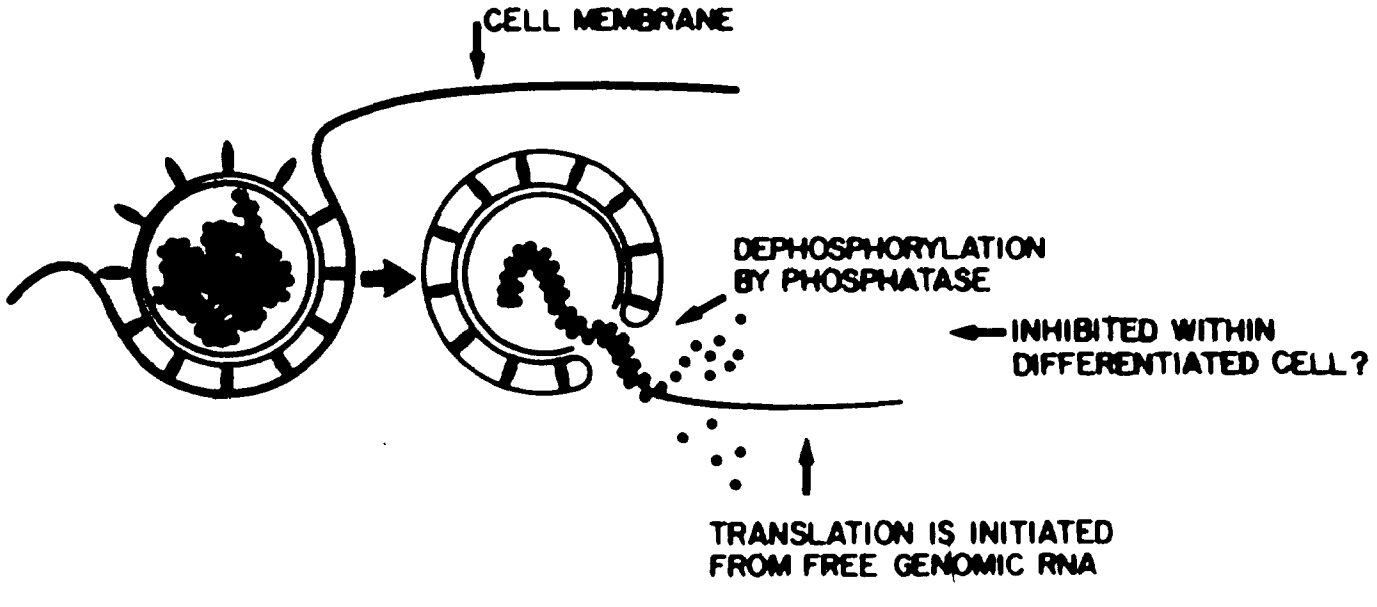
transcription in VSV (Kingsford and Emerson, 1980; Hsu et al., 1982). The possibility that dephosphorylation of NC enclosing JHMV RNA might be involved in the uncoating process prompted us to examine any consequence which induction of oligodendrocyte differentiation may have on cellular phosphatases. Directly pertinent to this study is the evidence of Leis et al (1984), that dephosphorylation of an avian retrovirus NC protein, pp12, reduces approximately 100 fold the binding affinity between this NC component and the RNA genome, suggesting that a similar dephosphorylation mechanism may be involved in the uncoating of JHMV RNA. This hypothesis is supported by Western blot evidence which reveals that during penetration the JHMV NC of inoculum virions is rapidly converted from a 56K into a 50K component (Coulter-Mackie et al., 1985). Since these two polypeptides of CV appear to be identical by 2-D tryptic peptide mapping (Cheley and Anderson, 1981; Sidell, 1982) reduction in apparent MW could be due to an alteration in charge of the polypeptide, perhaps due to removal of phosphorous. It remains to be resolved whether processing into p50 is initiated by dephosphorylation or proteolytic cleavage, as others have suggested (Cheley and Anderson, 1981), or a combination of the two events. On the basis of 2-D protein analyses it is likely that p56 is dephosphorylated before undergoing proteolytic modification to form the next product of processing, p40, shown to be related to p56 by pulse chase experiments and peptide mapping. Whether dephosphorylation or proteolysis to p50 occurs is difficult to prove with [^{35}S] methionine labelled material because the NC protein of JHMV possesses 86 amino acids on the carboxy side of the last methionine

residue (Skinner and Sidell, 1983), and there are in this stretch 13 potential cleavage sites for trypsin, whereby unlabelled tryptic-fragments could be formed but their loss would not be detected by peptide mapping. Evidence for proteolysis to p40 is the inability to detect this product in extracts of JHMV infected cells by either Western blot analysis with mouse monoclonal anti-NC antibodies (Coulter-Mackie et al., 1985) or immunoprecipitation of [^{35}S] labelled JHMV polypeptides (not shown) indicating that the reactive epitope is missing. Furthermore, failure of p40 to bind nucleic acids indicates that the RNA binding domain has been lost. When extracts from oligodendrocytes pretreated with 1mM dbcAMP or 8-Br cAMP are incubated with NC, processing to p40 is inhibited demonstrating that the requisite factors are suppressed upon differentiation. The suppression appears to coincide with both a reduction in the activity of phosphoserine-specific Fe^{++} dependent acid phosphatase(s), similar in type to that shown to exist in murine and porcine tissues (Paigen, 1958; Paigen and Griffiths, 1958; Schlosnagle et al., 1976) and a block at an early stage of JHMV expression. Taken together this evidence strengthens the hypothesis that CV uncoating is normally facilitated by phosphatase-catalysed NC dephosphorylation, a step in processing affected by differentiation. Although the present data on enzyme characterization and substrate specificity are only preliminary and sketchy, the available evidence was used to develop a provocative hypothesis about regulation of uncoating via cellular phosphatase(s) as the basis for explaining the observed cAMP-related inhibition of

JHMV replication in primary oligodendrocytes. The hypothesis is presented in Figure 21 as a diagrammatic depiction of events during CV uncoating within oligodendrocytes.

The importance of protein phosphatases as mediators of cellular regulation has recently received much attention (Cohen, 1982 Ingebritsen and Cohen, 1983; Sparks and Brautigan, 1986). Modulation of protein phosphatases and activation of phosphatase inhibitors has been associated with increases in intracellular levels of cAMP, as in the inhibition of protein synthesis caused by the arrest of the phosphatase-mediated recycling of eukaryotic initiation factor 2 (Ingebritsen and Cohen, 1983). Other examples of phosphatase-related regulation in cellular metabolism are listed in the review of Ingebritsen and Cohen (1983). Since virus replication depends on an appropriation of the metabolic machinery of the host it is not surprising that expression of specific viral functions may be altered or abrogated when cells undergo differentiation. This appears to be the state prevailing during oligodendrocyte differentiation, in which numerous metabolic changes are known to occur (McCarthy and de Vellis, 1980; Barbarese and Pfeiffer, 1981; Pfeiffer et al., 1981; McMorris, 1983; Wernicke and Volpe, 1986). Some of these changes, perhaps involving modulation of phosphatases, might bring about a repression of CV replication by impeding the uncoating process.

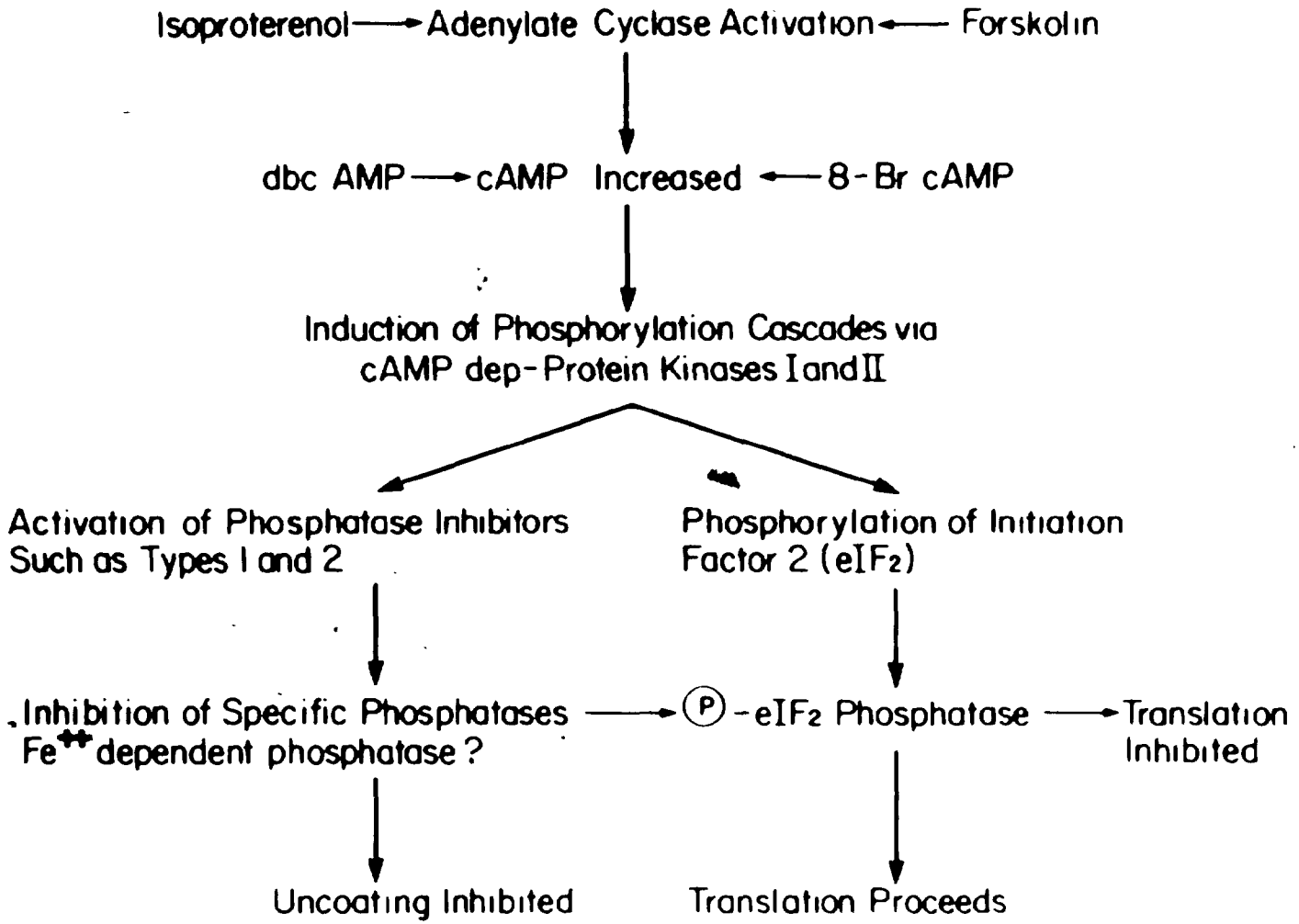
Figure 21 A diagramatic representation of hypothetical events connected with CV uncoating. Dephosphorylation of NC protein is proposed as a necessary event for initiation of processing of NC to facilitate conformational relaxation of the genome and/or release of NC from the genome to allow the expression of subsequent virus specified functions.



EARLY CELL-VIRUS INTERACTIONS

Based on established and presumed information about the influence of cAMP on differentiation, offered as a framework for future investigation is the chart in Figure 22 in which cAMP-induced metabolic changes are interrelated with CV expression, with particular attention given to the role of protein phosphatases.

Figure 22 Conceptualization of the interrelationship between metabolic events following activation of the adenylate cyclase system and CV replication. The chart illustrates two possible mechanisms through which CV replication might be arrested following differentiation of oligodendrocytes. Inhibition of phosphatase activity towards phosphorylated eukaryotic initiation factor 2 (Ingebristen and Cohen, 1983) could contribute to the arrest of translation of virus-specific mRNA. However, no evidence pertaining to this idea was obtained. Inhibition of Fe^{++}-dependent phosphatase activity, for which some evidence is furnished, implies that dephosphorylation of the NC is arrested, preventing uncoating and hence the sequelae of virus-specified functions.


Isoproterenol
Adenylate Cyclase Activation
Forskolin
dbc AMP
cAMP Increased
8-Br cAMP
Induction of Phosphorylation Cascades via cAMP dep-Protein Kinases I and II
Activation of Phosphatase Inhibitors Such as Types 1 and 2
Phosphorylation of Initiation Factor 2 (eIF2)
Inhibition of Specific Phosphatases
Fe++ dependent phosphatase?
(P)-eIF2 Phosphatase
Translation Inhibited
Uncoating Inhibited
Translation Proceeds

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differences in titre are attributed to the minor changes in density of oligodendrocytes in culture which were shown previously to profoundly influence JHMV production (Beushausen and Dales, 1985).

Infection of rat neural cells by CV is characterized by an unambiguous tropism of strain MHV3 for astrocytes and JHMV for oligodendrocytes (Beushausen and Dales, 1985). In this system it was demonstrated that a 48 hr. pretreatment of oligodendrocytes with dbcAMP completely abolished JHMV replication, whereas it only marginally affected the replication of MHV3 in astrocytes. To determine whether a prolonged exposure of astrocytes would be more effective, primary astrocyte cultures were pretreated with 1 mM dbcAMP and the titres of MHV3 produced determined. It is evident from Table 9 that pretreatment of astrocytes for as long as 5 days did not diminish the ability of these cells to produce virus. This observation is consistent with the view that elevation of cAMP affects differentially CV replication in astrocytes and oligodendrocytes.

To examine whether cAMP influences CV replication in other cell types in which differentiation can be induced, L6 rat myoblasts and the L6 mutant subline, JRU_5 were exposed to 1 mM dbcAMP. It is evident from the results in Table 10 that pretreatment of both types of myoblasts arrested production of MHV3 and JHMV. By comparison, the untreated controls became persistently infected, yielding within 3 days titres in excess of 10^3 PFU/ml, in agreement with Lucas et al. (1978). It is probably not coincidental that treatment with dbcAMP affects CV production in myoblasts and oligodendrocytes in a similar manner, because it has been shown that drugs which stimulate the