IN VIVO AND IN VITRO MODELS OF DEMYELINATING DISEASE XXI: RELATIONSHIP
BETWEEN DIFFERENTIATION OF RAT OLIGODENDROCYTES AND CONTROL OF JHMV
REPLICATION

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INTRODUCTION

Coronavirus (CV) infection in the central nervous system (CNS) of rodents, first described by Bailey et al. (1), is under investigation by many laboratories as a model of virus-induced demyelinating disease (10,25,38). One of the most intriguing factors influencing the disease process in rats is the development of an age-related resistance to the neurotropic CV strain JHM virus (JHMV) which becomes manifested at about the time of weaning, when the animal is 3 weeks old and myelination of the CNS is being completed (37). When tested in vitro in primary explants of neural cells from the neonatal rat CNS, replication of JHMV can be initiated in recently established oligodendrocyte cultures but is repressed in 3 week old cultures, coincident with their "time-clock" of differentiation. Experimentally manipulated differentiation of cultured oligodendrocytes by treatment with cAMP analogues such as N⁶, 2'-O-dibutyryl-adenosine 3':5'-cyclic monphosphate (dbcAMP) or metabolites affecting the levels of cAMP also results in suppression of JHMV replication (3). This implies that metabolic events leading to the development towards the terminally differentiated cell may be involved in regulating virus expression. Control over virus expression by regulation of cAMP levels is not unique to either the CV or the oligodendrocyte: it has been shown to occur in other virus-cell systems, including infections of neuronal and non-neuronal cells by measles virus (24,31,46), and rat glial cells by rubella virus (41). Although to date the control over virus expression in these systems has not been elucidated a relationship

to adenylate-cyclase and related metabolic pathways, the topic of this study, is suspected.

Results and Discussion

(a) Intracellular cAMP levels and Virus Replication. Treatment of primary explants of rat oligodendrocytes with two analogues of 3':5'cAMP, known to enhance differentiation, affected formation of CV progeny. As shown in Table 1, pretreatment with dbcAMP and 8 bromoadenosine-3':5'-cyclic monophosphate (8-Br cAMP) entirely blocked the replication of JHMV. Likewise, compounds which bring about increased intracellular cAMP levels by indirect action on the adenylate cyclase system, including isoproterenol and forskolin, suppressed production of JHMV (Table 1). Lack of involvement of cGMP was evident after treatment of oligodendrocytes with the analogue dbcGMP, which had no effect on virus

Table 1
Effect of Differentiation Inducers on Coronavirus Replication
in Primary Rat Oligodendrocytes

Treatment	Titres *on Days After Infection		
	1	3	10
Control	1.0	1.0	100
1mM dbcAMP	0	0	0
1mM 8-Br cAMP	0	0	0**
20uM Isoproterenol	1.75	0	500
50uM Isoproterenol	0.15	0	0
20uM Forskolin	0.3	0.05	70
50uM Forskolin	0.25	0	0
100uM Forskolin	0.8	0	0

*Note: All titres x 10^2 PFU/ml

Cell density: $2-3 \times 10^5$ cells/well

**sampled 9 days after infection

production, indicating that the virus is suppressed when metabolism related specifically to cAMP is perturbed.

In our system, contrary to the findings reported recently by others (20), infection of rat neural cells by CV is characterized by an unambiguous tropism of strain MHV₃ for astrocytes and JHMV for oligodendrocytes (3). When oligodendrocytes are pretreated for 48 h with dbcAMP JHMV replication does not occur whereas similar treatment affects only marginally MHV₃ production by astrocytes, demonstrating that elevation of cAMP affects differentially CV replication in oligodendrocytes and astrocytes. Influence of cAMP in other cell types in which differentiation can be induced was tested in L6 rat myoblasts and mutant sublines. In these cells dbcAMP pretreatment blocked production of both JHMV and MHV₃ (Table 2) whereas untreated controls became persistently infected with both viruses, as shown by us previously (19). It is probably not coincidental that dbcAMP affects CV replication in myoblasts and oligodendrocytes in a similar manner, because it has been shown that drugs which stimulate formation of cAMP also promote differentiation of myoblasts (47).

(b) 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. Increases in intracellular levels of cAMP produce changes in the enzymatic activities of the cAMP-dependent protein kinases, PKI and PKII, which are involved in initiating the phosphorylation of various intracellular substrates (7,17). In the nervous system, the role of cAMP is presumed to be fundamental for neurotransmission and neuronal/glial cell communication (9,34), two functions generally presumed to be mediated through the activation of PKII, the predominant activity in brain tissue (26,44). We assessed the relative activities of PKI and PKII in untreated neural cell material. For this purpose, cell-free extracts of astrocytes and oligodendrocytes were prepared, then separated into fractions by DEAE-cellulose column chromatography, and assayed for [³H]-cAMP binding kinase activity. It was found that neural cells are conspicuously devoid of PKI, consistent with absence of the enzyme from isolated neural tissue (44). The predominant isozyme found by us in primary cultures of rat astrocytes and oligodendrocytes is PKII. For comparison, PK activity assays in L6 myoblasts showed considerable amounts of PKI as well as PKII, in confirmation of previous data (32). Following treatment with dbcAMP the activity of PKI in L6 myoblasts was diminished or eliminated but that of PKII was not affected. Similarly, there was no effect on PKII levels after 48 hour treatment of neural cell cultures with dbcAMP. 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⁻ JRU₅ mutant myoblasts as well as in the PKI⁻ neural cells (Table 2).

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 physiochemical properties. Consequently, it has been suggested that the enzyme specificity of PKs resides in the R component (16). 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 [³H] cAMP or the covalent binding to the photoaffinity analogue 8-azido-[³²P] cAMP (8-A-cAMP) was determined. In the case of L6 myoblasts treatment for 40 hours 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 (data not shown). We also monitored the levels of R in astrocytes and oligodendrocytes by photo affinity labeling of cytosol extracts with 8-A-cAMP, which binds specifically to polypeptides of MW 47,000 (47 K) and 52,000 (52 K), known to be RI and RII, respectively (32,42). Treatment of oligodendrocytes with 1mM dbcAMP for 48 hours caused a marked increase in the amount of RI despite the absence of PKI in these cells, but the quantity of RII changed very little (data not shown).

The relationship between duration of treatment with 1mM dbcAMP and the levels of RI and RII in astrocytes and oligodendrocytes was also assessed. The data, presented in Figure 1, show the relative amounts of RI, as estimated from the area under each peak in the densitometric tracing made from the original autoradiogram. In astrocytes treated for 3 days there was a 2.43 fold increase 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. With RII, dbcAMP caused a maximal increase after 3 days to a level only 1.2 fold greater than in the controls. By the fifth day of treatment the RII concentration declined to 0.61 that of the controls.

In the case of oligodendrocytes, dbcAMP caused a rapid increase in free RI, so that within 24 hours this component was about 3.5 times more abundant (Fig. 1) 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 by the third day post treatment to 1.48 that in the controls and remained at this level thereafter. As a test of the specificity of dbcAMP, treatment with 1mM dbcGMP for 48 hr. did not cause any change in RI of oligodendrocytes.

Table 2
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	MHV ₃
L2 Mouse Fibroblasts	Control	+-	++	+	ND	+++	+++
	1mM dbcAMP	-	++	++	ND	+++	+++
Rat Astrocytes	Control	-	++	+	++	-	+++
	1mM dbcAMP	-	++	++	++	-	+++
Rat Oligodendrocytes	Control	-	++	+	++	+++	-
	1mM dbcAMP	-	++	++++	+++	-	-
L6 Rat Myoblasts	Control	+++	++	*	++	+	+
	1mM dbcAMP	-	++	**	++	-	-

*increases with time within the cell

**increase observed only in L6 subline JRU₅

4+ extensive replication 10⁵ PFU/ml; 3+ less extensive replication 10⁴ PFU/ml; 2+ yields 10³ PFU/ml; 1+ yields 10² PFU/ml

Figure 1. 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-A-cAMP. In (A) a densitometer 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 subunit RI. Absorbance units have been normalized to the band of greatest density, (oligodendrocytes, 5 days post treatment).

Exposure of myoblasts and neural cells to 8-Br cAMP, which also induces cell differentiation, suppressed JHMV replication in oligodendrocytes and L6 cells (Table 1). However, treatment with 1 mM 8-Br cAMP caused a decrease of RI in both cell types, presumably due to a destabilization and increased turnover of RI caused by the binding of the bromine analogue (11,39) (data not shown).

The present observations summarized in Table 2, when taken together support the idea that inhibition of CV replication in oligodendrocytes and L6 myoblasts is affected through activation of the adenylate cyclase system,

manifested in the modulation of free RI. In either instance, whether it be by an increase or more rapid turnover of the molecule, the catalytic subunit of PKI is free to effect induction of a kinase cascade presumed to be involved in differentiation and may contribute to inhibition of CV replication.

(c) 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. Adsorption of JHMV to either astrocytes or oligodendrocytes was unaffected by prior exposure to 1 mM dbcAMP (data now shown). It should be noted from this finding that ability of JHMV to become adsorbed onto astrocytes revealed that specificities in tropism, whereby JHMV fails to replicate in rat astrocytes (3), are not related to the absence of the relevant receptors.

Rates of JHMV internalization by oligodendrocytes, measured as clearance of cell-associated virus from the external surface, were also unimpaired, by prior treatment with 1 mM dbcAMP (data not shown).

Occurrence of viral-related syntheses in dbcAMP-treated oligodendrocytes, was analyzed: JHMV RNA production by means of dot blots and expression of antigens using antibodies employing the indirect immunofluorescence technique. The tests failed to detect either transcription or translation (data not shown), indicating that expression of JHMV within differentiated oligodendrocytes was interrupted at some stage subsequent to virion internalization and suggesting that this inhibition could be connected with the virus uncoating process.

The plus stranded RNA genome of CV is, by itself, infectious (43), implying that viral functions may commence after the genome has been divested of the nucleocapsid protein coat, in which it is wrapped when inside the virus envelope. The presence of phosphorylated residues in the nucleocapsid protein (NC) suggested the possibility that uncoating of the RNA might be facilitated by a host phosphoprotein phosphatase enzyme, related to the cAMP-dependent protein kinase cascades. Therefore, repression of such an enzyme upon oligodendrocyte differentiation might underly the arrest of JHMV expression. This notion is in accord with the view of Ingebritsen and Cohen (13), that protein phosphatases and kinases are important targets for cellular regulation. Based on evidence for penetration of CV via endosomes, organelles

which possess very high activities of acid phosphatase (8,28), we examined the relationship between differentiation and acid phosphatases of oligodendrocytes.

Determination of optimum conditions for the phosphatases presumed to dephosphorylate the substrate of specific interest here, the NC protein of CV, revealed that acid phosphatase(s) in the particulate fraction of oligodendrocytes have the capability to dephosphorylate isotopically labeled NC, shown previously to be phosphorylated exclusively on serine residues (40). After mixing with cell extracts the [^{35}S]-NC protein underwent processing, as evident from Figure 2. 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 Figs. 2A vs B 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 to intermediates, primarily into p50 and p40, as illustrated in Figs. 2C 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 1D (Fig. 3) and 2-D (data not shown) SDS-PAGE analyses on [^{35}S] and [^{32}P] labeled extracts of JHMV infected L-2 cells. It is possible that the p40 product may have been derived by proteolytic cleavage in conjunction with or soon after dephosphorylation of the p56 NC.

The relationship between treatment of oligodendrocytes with differentiation inducers and activity of NC-related processing in the particulate fraction was also examined. Extracts derived from untreated cultures produced more efficient processing of the [^{32}P] NC protein under conditions optimal for Fe^{++} dependent phosphatase activity than did comparable extracts from cultures treated with either dbcAMP or 8-Br cAMP, as judged by the quantity of p40 appearing in Figure 3. These observations are consistent with the view that acidic phosphoprotein phosphatase(s) acts in dephosphorylation of the NC during normal CV penetration and uncoating, and that this process is repressed when oligodendrocytes are driven towards differentiation.

Figure 2. 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^{++} . 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. In C the reaction was carried out without Fe^{++} ; in D processing to p40 was evident when Fe^{++} was added.

(d) Affinity for Binding Nucleic Acid by the Nucleocapsid Protein and its Products. Robbins et al. (30) 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 which fits our hypothesis regarding the uncoating of CV, was tested using JHMV RNA labeled with [^{32}P] and cDNA labeled with [^{35}S]. 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 labeling with [^{35}S] methionine in pulse chase experiments. The SDS-PAGE analysis of extracts, illustrated in Figure 4, showed that NC p56 was partially converted into p50, a related protein

Figure 3. 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 . 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.

indistinguishable from p56 by [^{35}S] 2-D tryptic peptide mapping (4,5) and also into p40, a processed product of NC. It is evident from Figure 3 that p56 was the prominent JHMV protein binding nucleic acid, confirming the findings of Robbins et al. (30). It may be highly significant that neither the RNA or cDNA was adsorbed to the polypeptide band at the position of p40, indicating that this component lacked the relevant domain for binding nucleic acid. This evidence further supports the view that CV uncoating depends on host controlled release of NC from the viral genome so as to permit the freed RNA to participate in the replication cycle.

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 phosphatases, two classes of enzymes which participate in the regulation of normal cellular functions. This idea may have considerable bearing on

Figure 4. 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 h, both the p56 NC and its products p50 and p40 are prominent (lane 6). It should be noted that due to the early labelling of the monolayer culture in the asynchronous infection many host polypeptides were also labelled.

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 transcription (12,14,15), while dephosphorylation of NC enclosing JHMV RNA might be involved in the uncoating process. Directly pertinent to this study is the evidence of Leis et al. (18), that dephosphorylation of an avian retrovirus NC protein, pp12, reduces approximately 100 fold the binding affinity between this NC component and

the RNA genome. On the basis of our 2-D protein analyses we favour the notion 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. Failure of p40 to bind nucleic acids indicates that upon processing the RNA binding domain has been lost. When extracts from oligodendrocytes pretreated with 1 mM dbcAMP or 8-Br cAMP are added to the NC, processing is inhibited, demonstrating that the requisite factors are suppressed upon differentiation. The suppression appears to coincide with both a reduction in phosphoserine-specific Fe^{++} dependent acid phosphatase(s) activity, similar in type to that shown to exist in murine and porcine tissues (27,28,33) and a block at an early stage of JHMV expression. Taken together this evidence strengthens our hypothesis presented diagrammatically in Figure 5, that CV uncoating is

Figure 5. Diagrammatic 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.

normally facilitated by phosphatase-catalyzed NC dephosphorylation, a step in processing affected by differentiation. During oligodendrocyte differentiation, in which numerous metabolic changes are known to occur (2,5,21,23,29,45), perhaps those involving modulation of phosphatases bring about a repression of CV replication by impeding the uncoating process.

Based on established and presumed information about the influence of cAMP on differentiation we offer as a framework for future investigation the chart in Figure 6, in which cAMP-induced metabolic changes are interrelated with CV expression, with particular attention on the role of protein phosphatases.

Figure 6. 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. Loss of phosphatase activity towards phosphorylated eukaryotic initiation factor 2 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.

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