If Dip-2$^-$ is a regulatory mutant preventing synthesis of a structural protein, no enzyme activity would be expected. However, Dip-2 was expressed in hybrids, presumably by the RAG genome. These results and the formation of a hybrid enzyme in human-mouse cell hybrids and in in vitro mixtures indicate that the Dip-2 deficiency is not a regulatory mutant.

The fact that in vivo and in vitro hybridization occurred between mouse inactive peptidase Dip-2 and human active Pep-A indicated (i) a structural mutation for the *Dip-2* locus, (ii) structural homologies for the human and mouse enzymes, (iii) that the murine peptidase mutation did not eliminate subunit synthesis, and (iv) that the mutation did not eliminate the ability of inactive Dip-2 subunits to dimerize with the human gene product and form an active enzyme. The hybrid enzyme possessed the substrate affinities of human Pep-A and mouse Dip-2, and the staining intensities indicated that the hybrid enzyme was not less active than a hybrid enzyme composed of normal subunits.

Interspecific hybrid enzymes for several enzymes have been described in human-mouse cell hybrids (*6, 15*), and no restrictions have been described in the formation of heteropolymers. Thus, somatic cell hybrids may prove important in the study of human biochemical diseases that affect specific polymeric enzymes. The formation of hybrid enzymes with normal mouse and defective human components, and especially the formation of electrophoretically different hybrid enzymes, could provide information on genetic heterogeneity, subunit structure, structural alterations, and substrate specificities of mutant human enzymes.

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30 May 1972

Virions from Progressive Multifocal Leukoencephalopathy: Rapid Serological Identification by Electron Microscopy

Abstract. *Virions were extracted directly from the brain of a patient with progressive multifocal leukoencephalopathy (PML). They were treated with antiserum to SV40, with rabbit antiserum to previous PML isolates, or with serum from another patient with the same disease and observed directly by electron microscopy. This procedure could be used for the rapid identification of the antigenic nature of virions in cases of PML.*

Progressive multifocal leukoencephalopathy (PML) is a subacute human demyelinating disease in which large numbers of papovavirus-like particles have been repeatedly found by electron microscopy (*1*). In the past year virus has been isolated from the brains of three patients with this disease (*2, 3*). Padgett *et al.* (*2*) inoculated a homogenate from brain tissue of a patient with PML into monolayers of human fetal brain cells and isolated a virus; this agent appeared distinct from mouse polyoma virus, simian virus 40 (SV40), or human papilloma virus by immunofluorescent staining. Weiner *et al.* (*3*) using dispersion cultures of human cells (derived from the patients' brains) fused to monkey cells [primary African green monkey kidney (PAGMK)] recovered viruses from two patients with PML. A close serological relation between the virus isolated by Weiner *et al.* and SV40 virus was demonstrated.

The isolation of new agents in cell culture systems always raises the possibility of contamination, and the isolation of an agent in simian cells with the antigenic phenotype of a simian virus raises the possibility that recombination of the agent with latent agents has occurred (*4*). Therefore, a method of serologic identification of virions extracted directly from diseased tissues would provide a valuable adjunct to cell culture methods.

Table 1. Serologic interaction of virions from brain and from related viruses; +, definite aggregation; —, no agglutination; space, not done.

Serums	Virions extracted from brain (patient A)	SV40	Previous virus isolates in PAGMK		Polyoma virus
			Patient A	Patient B	
Monkey antiserum to SV40	+	+	+	+	—
Horse antiserum to SV40	+	+			
Rabbit antiserum to virus isolated from patient A	+		+	+	—
Rabbit antiserum to virus isolated from patient B	+		+	+	—
Serum from patient A	—	—	—	—	
Serum from patient B	+	+	+	+	—
Rabbit antiserum to polyoma	—	—			+
Control serums					
Monkey serum before immunization to SV40	—	—	—	—	
Horse serum before immunization to SV40	—	—		—	
Rabbit antiserum to PAGMK cells	—		—	—	
Normal human serum		—		—	

The electron microscopic visualization of virus-antibody agglutination has been used to detect small amounts of antibody and to show serologic cross-reactivity of viruses (*5*). In our study electron microscopy was utilized for serologic identification of virions extracted directly from human brain.

Electron microscopy of the white matter from the brain of a patient with PML (*6*) revealed virions. The white matter was rapidly frozen and thawed three times, minced, and ground in a chilled mortar. A homogenate (10 percent) was made in phosphate-buffered saline (*p*H 7.4), containing 1 percent sodium deoxycholate and 0.025 percent trypsin; it was stirred for 30 minutes at 37°C, and 1.5 ml portions were layered on 6 ml of 5 percent sucrose in 0.02*M* tris buffer (*p*H 7.5) and centrifuged at 100,000*g* for 2 hours. The pellets were resuspended in 0.001*M* tris buffer (*p*H 7.8) containing 0.01 percent bovine serum albumin fraction V and centrifuged at 1000*g* for 30 minutes (*2*, *7*). The supernatant was treated with Freon to separate bound virions from membranes (*8*). Other virus preparations included the two PML agents previously isolated in PAGMK (from patient B, $10^{9.4}$ infectious units per milliliter, and from patient A, $10^{7.2}$ infectious units per milliliter), SV40 (10^{8} infectious units per milliliter in PAGMK), and polyoma virus ($10^{5.3}$ infectious units per milliliter in primary mouse embryo fibroblasts. The homogenates of brain and the tissue culture preparations contained enough virions so that further purification and concentration were unnecessary.

Serum from patient B (neutralizing antibody titer to SV40 of 1:640 and to the PML isolate of 1:1280) (*9*), rabbit antiserum to the PAGMK isolates (neutralizing antibody titer of 1:640 to PML isolates), antiserums before and after infection with SV40 from monkey (1:640) and horses (1:320) were also used. Serums were inactivated at 56°C for 30 minutes, diluted with Hanks balanced salt solution and centrifuged for 1 hour at 100,000*g* to clarify.

Virus-antibody complexes were prepared by mixing 0.1 ml of undiluted virus suspension with 0.2 ml of serum (diluted 1:10) and 0.7 ml of Hanks balanced salt solution, incubating for 1 hour at 37°C, and placing at 4°C overnight (*5*). The mixture was then centrifuged at 15,000*g* for 30 minutes, and portions of the bottom 0.3 ml were placed for about 15 seconds on 200-mesh copper grids (previously coated with Parlodion and carbonized). A drop of 2 percent phosphotungstic acid was then added for 30 seconds. The fluid was drawn off with filter paper, and the grid was examined immediately in a Philips 200 electron microscope.

Virus-antibody complexes were observed as virions separated and connected by a latticework of antibody molecules (Fig. 1) or as individual virions with attached antibody molecules. The antibody appeared as fine filamentous structures. Virions without specific antiserum appeared as clear, well-defined individual particles (Fig. 2). Virions sometimes aggregated along membranes, but such aggregates were considered to be preparative artifacts. The interactions of the virions and serums are summarized in Table 1. Of particular interest is the reaction of virions extracted by physical means directly from the brain of patient A with monkey and horse antiserums to SV40, and rabbit antiserum to previous tissue culture isolates. Serum from patient A did not react with virions from her brain as well as with other viruses; this result confirms previous neutralization tests that failed to demonstrate antibody in this serum. However, serum of patient B with PML did agglutinate these virions as well as previous isolates and SV40.

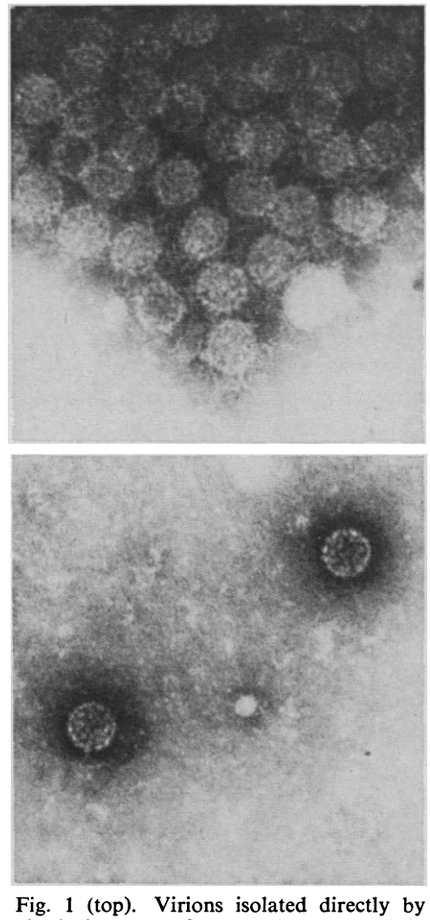

Fig. 1 (top). Virions isolated directly by physical means from patient A's brain were reacted with the serum of patient B. Agglutination of the virus is apparent with the antibody molecules appearing as filamentous white lines connecting the virions (× 142,000). Fig. 2 (bottom). Virions isolated from brain of patient A were incubated with a pre-immunization serum containing no specific antibody to the virus. Virions show no attached antibody molecules and capsids are clearly outlined (× 150,000).

The reactions demonstrate that the particles in the patient's brain were antigenically related to SV40. The reactions of antiserum with virions from brain confirm the isolation studies and indicate that the SV40-like antigenic phenotype did not result from recombination with latent simian agents in cell cultures. The lack of cross-reaction with polyoma virus further confirms the specificity of this method.

In that two antigenically distinct papovaviruses have been associated with PML, this disease does not appear to be due to a single agent. The isolation and serological identification of agents from brains of patients with PML by means of either explant methods or human fetal brain cultures is a time-consuming procedure requiring several months. In contrast, immune electron microscopy may have the potential to distinguish very rapidly antigenically distinct virions extracted directly from brains of patients with PML.

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9. Patient B [case 1 in (3)] had a 21-month history of PML (with no apparent underlying process) which ended in death. The serum of this patient showed consistently elevated concentration of SV40 antibody.
10. We thank Drs. W. Flor and K. Shah for assistance. Supported by PHS grants NS 08839, NS 08997, and NS 08963. L.P.W. is the recipient of career development award NS 50274.

5 May 1972; revised 7 July 1972

Chromatid Breakage: Differential Effect of Inhibitors of DNA Synthesis during G_2 Phase

Abstract. *The cell cycle specificity of chromatid breakage induced by inhibitors of DNA synthesis depends on the mechanism of drug action. 5-Hydroxy-2-formylpyridine thiosemicarbazone, hydroxyurea, and guanazole, compounds that inhibit ribonucleotide reductase, do not cause chromatid breakage during G_2 phase. In contrast, two active antitumor agents, arabinosylcytosine and 5-azacytidine, which are either incorporated into polynucleotides or affect DNA polymerase, produce chromatid breakage during G_2 phase. All of these agents except guanazole also induce breakage in S phase.*

Because of the demonstration by Benedict *et al.* (*1*) that the antitumor agent arabinosylcytosine (ara-C) induces chromatid breakage in the G_2 phase of the cell cycle, other inhibitors of DNA synthesis were studied for their effect during G_2 phase. These studies indicate that the induction of chromatid breakage during G_2 phase is dependent upon the particular drug employed, and that breakage is not a property of all inhibitors of DNA synthesis. This breakage was the major chromatid aberration produced in G_2 phase by the drugs.

Stationary cultures of the hamster fibroblast line Don-C (T_C, 13 hours; G_1, 3.6 hours; S, 6.2 hours; G_2, 2.2 hours; and M, 0.7 hour) were exposed to ara-C, 5-azacytidine (aza-C), 5-hydroxy-2-formylpyridine thiosemicarbazone (5-HP), hydroxyurea, or guanazole; dosages and durations are shown in Table 1. Metaphase cells exposed to drug during G_2 phase were obtained by adding Colcemid (0.06 μg/ml) to synchronous cultures 2.5 hours after the DNA inhibitor was added. Colcemid was added 4.5 hours after the drug if metaphase cells treated in S phase were desired. Metaphase cells were prepared for chromosomal analysis as described (*1*).

The results are tabulated in Table 1 together with some information on the mechanism of action of these inhibitors of DNA synthesis. The compounds ara-C and aza-C both cause chromatid breakage in S and G_2 phases; ara-C primarily affects the activity of DNA polymerases (*2*). Although the target enzyme for aza-C is uncertain, the drug is incorporated into DNA and RNA polynucleotides, replacing 20 to 30 percent of cytidine in bacterial systems (*3*), and it does not affect DNA polymerase (*3*). Hydroxyurea and 5-HP (*4*), both known to be inhibitors of ribonucleotide diphosphate reductase, do not produce chromatid breakage during G_2 phase but do so during S phase. Guanazole (*5*), another inhibitor of ribonucleotide reductase, does not cause breakage in either S or G_2 phase, even at doses that produce inhibition of cell proliferation.

Several kinds of DNA polymerases have been described (*6*). Studies in bacterial (*2*) and animal (*7*) systems have indicated that ara-C does not inhibit DNA polymerase 1, the enzyme probably involved in dark repair of DNA breakage induced by ultraviolet radiation. These data suggest that chromatid breakage induced by ara-C during G_2 phase is related to inhibition of scheduled DNA synthesis. These results are consistent with our previous evidence that such breakage is reduced by treatment with ultraviolet radiation before ara-C is given (*8*). Chromatid breakage induced by aza-C may be the result of incorporation into DNA polynucleotides.

Table 1. Differential effects of inhibitors of DNA synthesis on chromatid breakage.

Target enzyme	Incorporation into DNA (%)	Dose (μg/ml)	Phase exposed	Duration of exposure (hour)	Metaphase cells (%) in which breaks per cell = 0	1–4	5–9	10+
		Control						
		None			98	2	0	0
		Ara-C						
DNA polymerases	0.1	10	G_2	0.5	72	28	0	0
			S		77	23	0	0
		Aza-C						
Unknown	20–30	10	G_2	1.0	72	20	4	4
			S		46	40	6	8
		5-HP						
Ribonucleotide reductase	None	100	G_2	1.0	98	2	0	0
			S		64	24	6	6
		Hydroxyurea						
Ribonucleotide reductase	None	100	G_2	1.0	94	6	0	0
			S		60	30	4	6
		Guanazole						
Ribonucleotide reductase	None	1000	G_2	1.0	96	4	0	0
			S		98	2	0	0

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9. Supported by NIH grant CA 11050 and American Cancer Society grant T-493; M.K. is a Scholar, Leukemia Society of America. We thank N. Rucker for expert technical assistance.

5 June 1972