THE APPEARANCE OF INFECTIOUS MATERIAL IN A CELL-FREE SYSTEM OBTAINED FROM INFLUENZA VIRUS-INFECTED CALF KIDNEY CELLS

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There is evidence that the replication of RNA viruses, and therefore of viral RNA, may be catalyzed by an RNA-primed enzyme (Reich et al, 1962). Such an enzyme has been demonstrated by Baltimore and Franklin (1962) in L-Cells infected with the RNA-containing Mengovirus and by Weissman, Simon and Ochoa (1963) in Escherchia coli infected with the RNA-containing phage MS-2. A preparation with properties similar to the above-mentioned preparations has been isolated from calf kidney cells infected with the RNA-containing Influenza virus and from chick embryonic fibroblasts infected with Newcastle's Disease virus. A detailed report of the properties of these two systems is in preparation.

Evidence will be presented in this report that a preparation obtained from Influenza virus-infected calf kidney cells leads to the appearance of infectious material.

METHODS

Monolayers of calf kidney cells were inoculated with 1 x 10^6 $TCID_{50}$ of Influenza Type B virus (Maryland strain) and incubated at 36° C for ten hours. The media was decanted, 5 ml. of 0.2% trypsin in 0.05% Versene added to the culture flasks and after 30 minutes the cell suspension was collected. The cells were removed from the suspension by centrifugation at 600 x g for ten minutes at room temperature. The cell sediment was washed three times with sterile 0.25 M Sucrose-0.005 M $MgCl_2$. Sterile procedures and solutions were employed throughout the experiment and all subsequent steps were carried

out at 5° C. The washed cells were suspended in five volumes of ice-cold Sucrose-$MgCl_2$, one volume of acid-washed glass beads (230-270 mesh) added and the mixture placed in a Virtis Homogenizer and homogenized at a setting of 70 for four minutes. The homogenate was centrifuged at 8,000 x g in a Spinco Model L ultracentrifuge for 15 minutes to remove the glass beads, cell debris and mitochondria. The supernatant fluid was centrifuged at 105,000 x g for 60 minutes. From the 105,000 x g sediment, preparations A, B and C were prepared using the following media: Media A, Sucrose-$MgCl_2$ with 10 ug. per ml. of DNAase; Media B, Sucrose-$MgCl_2$ with 10 ug. per ml. of DNAase and 10 ug. per ml. of RNAase; and Media C, Sucrose-$MgCl_2$ with 10 ug. per ml. of DNAase, 10 ug. per ml. of RNAase and 1000 $TCID_{50}$ per ml. of Influenza B virus. All three preparations were pre-incubated for 10 minutes at 37° C.

An aliquot of the 105,000 x g sediment was studied by electron microscopy and found to be free of intact cells and to contain microsomal particles.

The complete reaction mixture was similar to that of Weissman et al. (1963) and contained (in micromoles): Tris HCl buffer, pH 7.2, 40; $MgCl_2$, 3.0; mercaptoethylamine, 4.0; phosphoenolpyruvate, 2.0; pyruvate kinase, 70 ug.; ATP, CTP, GTP and UTP, each 0.2; sodium orthophosphate, 40; and preparation A, B or C (350 ug. protein) in a final volume of 0.4 ml. Incubation time was ten minutes at 37° C and zero time controls were run simultaneously with each preparation. Preparation blanks were prepared by substituting Sucrose-$MgCl_2$ for the incubation media. The reaction was terminated by freezing the mixture in a dry ice-acetone bath. Triplicates were run at each condition.

Three 0.1 ml. aliquots were removed from each sample and inoculated into calf kidney cell cultures and incubated at 36° C. After four days, aliquots were removed from the culture tubes and assayed for virus by the hemagglutinatic test with 0.5% guinea pig red blood cells and incubated at room temperature.

RESULTS

The results from the tests for infectivity are indicated in TABLE 1.

Table 1. Assay for Infectivity in a Cell-Free System Obtained from Influenza-Infected Calf Kidney Cells

Preparation	Pretreatment	Incubation Media	Time of Incubation (minutes)	Hemagglutination Assay for Virus*
A	DNAase	Sucrose-$MgCl_2$	0	0
			10	+
		Complete	0	0
			10	+
B	DNAase and RNAase	Sucrose-$MgCl_2$	0	0
			10	0
		Complete	0	0
			10	0
C	Virus, DNAase and RNAase	Sucrose-$MgCl_2$	0	+
			10	+
		Complete	0	+
			10	+
Sucrose-$MgCl_2$	None	Complete	0	0
			10	0

* 0 means hemagglutination negative
\+ means hemagglutination observed

Positive tests for infectious material were obtained in all experiments in which virus was added to the preparation, even though the preparation and virus were subjected to DNAase and RNAase treatment (Rows 9-12). Infectivity was also observed in the 10 minute incubated samples containing the preparation treated with DNAase (Rows 2 and 4) but not in the zero time controls (Rows 1 and 3). The appearance of infectious material in the incubated preparation blank (Row 2) could be due to the crude preparation containing some of the required substrate nucleotide triphosphates, particularly since the pellet was not washed. However, when the preparation was subjected to RNAase action all ability to produce infectious material was lost (Rows 5-8).

In another experiment with twice the usual amount of enzyme preparation, a serial dilution was performed on samples of the incubation mixture containing

complete media and preparation A taken at zero time and after 10 minutes. These diluted samples were inoculated into tissue cultures and after four days tested for hemagglutination as above. The results indicated that the titer of infectious material was higher in the incubated sample than in the zero time control, indicating an increase in the infectious material as a function of incubation in the complete system.

The infectious material found after the ten minute incubation of preparation A and the complete system was identified as Influenza virus by the hemagglutination inhibition test using specific hyperimmune rabbit sera.

DISCUSSION

The observation that infectious material was present after incubation of preparation A could be explained in a number of ways. Among the possibilities are: (1) that preparation A contained virus; (2) that preparation A contained inactive infectious material that became activated after incubation for 10 minutes, and (3) that viable infectious material was synthesized during the incubation.

That there was no gross contamination of the preparation with intact virus was indicated by the resistance of virus to RNAase action, as seen in the experiments with preparation C, and the sensitivity of preparation B to RNAase action. At present we cannot exclude the possibility that the preparation might contain infectious viral RNA, especially since we have seen some infectivity at zero time when larger amounts of preparation A are used. In fact, if RNA-primed RNA synthesis were to occur, one would expect the presence of viral RNA in the preparation which would serve as the templates. Experiments have been performed in this laboratory using these same preparations but measuring the incorporation of GTP-8-C^{14} into nucleic acid in the complete system instead of measuring infectivity. In these experiments RNAase treatment inhibited the incorporation of GTP-C^{14} into nucleic acid. A detailed report on these experiments is in preparation.

Similar results, i.e., demonstration of infectious material after the ten minute incubation with the complete system, have been obtained with a

preparation obtained from Newcastle's Disease virus - infected chick embryonic fibroblasts.

SUMMARY

The appearance of RNAase-sensitive infectious material after incubation of the DNAase-treated preparation obtained from influenza virus infected calf kidney cells was observed. This phenomena could be explained in at least two ways: (1) the unmasking of potentially infectious material in the preparation, or (2) the synthesis of infectious material during the incubation.

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