INHIBITION OF DNA SYNTHESIS BY ISOLATED LIVER NUCLEI FROM FROG VIRUS 3 INFECTED MICE.

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SUMMARY

Incorporation of [^{3}H] dTTP into acid insoluble material by liver nuclei from mice infected for 3 hours with 5 x 10^8 PFU of Frog Virus 3 was markedly decreased. The incorporation was noticeably stimulated by the addition of calf thymus DNA. It has been verified that the inhibition of incorporation by the nuclei from infected animals was not related to an increase of deoxyribonuclease activity and that the product of the reaction showed the properties of DNA.

Acute degenerative hepatitis induced in mice by frog virus 3 (FV_3), was shown to be of toxic origin since the virus was unable to multiply at 37°C (1 - 2). At the outset of the hepatitis, which led to the death of the animals within 18 to 24 hours, a drastic inhibition of the macromolecular metabolism of the liver, as well as considerable alterations of the nuclei of the hepatocytes could be demonstrated (3 - 4). Though the synthesis of RNA was principally affected, there was also a noticeable inhibition of thymidine incorporation in the liver of infected animals. Therefore it was of interest to study the DNA synthesis in a system consisting of isolated nuclei, which offered the possibility of be stimulated by exogenous primer DNA (6). In this paper, evidence is presented for an inhibition of DNA synthesis in isolated nuclei from the liver of FV_3 infected mice.

EXPERIMENTAL PROCEDURE

MATERIALS

Unlabeled deoxyribonucleosides triphosphates, ATP, were purchased from Sigma

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Chemical Co. Ribonuclease A and deoxyribonuclease were obtained from Worthington Biochemical Corp. Freehold, N.Y. [^{3}H] dTTP came from the Radiochemical Center Amersham U.K.

VIRUS

FV_3, kindly supplied by Dr A. GRANOFF, was grown in BHK_{21} cells at 26°C. Three days after infection, the medium (Eagle's MEM + 1% calf serum) was discarded ; the cells were collected in phosphate buffered saline and subjected to three cycles of sonication (3 x 1 min. MSE disintegrator). The cells were then centrifuged at 2,000 rpm and the supernatant used as crude virus suspension was kept at -80°C.

PREPARATION OF MICE LIVER NUCLEI (5)

Mice (I.C. strain) weighing from 18 to 25 g were inoculated intraperitoneally with 5 x 10^8 plaque-forming units (PFU) of FV_3. They were killed at different intervals by decapitation and their livers were immediatly homogenized in 10 ml per gram of liver of 2.2 M sucrose in buffer A (0.05 M Tris HCl buffer pH 8, 0.025 M KCl, 0.005 M 2-mercaptoethanol, 0.001 M EDTA and 0.005 M magnesium acetate). The homogenate was passed through four layers of cheesecloth and centrifuged at 40,000 g for 75 min. The pellets were taken up in 2 ml of buffer A containing 20% glycerol and frozen at -20°C.

ENZYME ASSAY

DNA polymerase assays were carried out in glass tubes in a final reaction volume of 125 µl (6). The components of the assay are given in the legends. After incubation, the samples were precipitated by addition of cold 10% trichloracetic acid. The precipitate was collected by filtration on Millipore filters (GSWP 02500), washed 5 times with 5 ml of cold 10% trichloracetic acid and counted in 10 ml of toluol 1 liter, PPO 2.5 diphenyl oxazole 4 g, dimethyl POPOP 1.4 - bis [2 - (4 methyl - 5 - phenyl oxazoly)] - benzene 0.4 g.

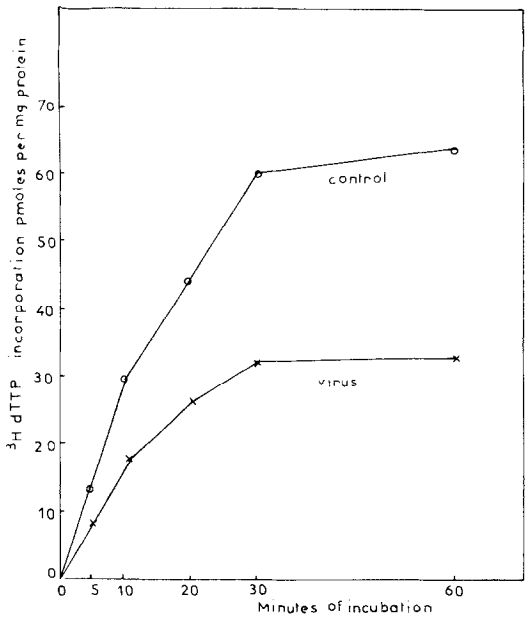

Figure 1 :

Kinetics of [^{3}H] dTTP incorporation into DNA by isolated nuclei from controls and mice inoculated intraperitoneally with 5 x 10^8 PFU of FV_3 for 3 hours. The standard reaction mixture contained in a final volume of 3 ml : 0.025 M Tris HCl buffer (pH 8), 0.005 M 2-mercaptoethanol, 0.005 M EDTA, 0.025 M KCl, 0.01 M magnesium acetate, 0.002 M ATP, 0.015 mM each of d ATP, d CTP, d GTP and [^{3}H] d TTP (specific activity 15 Ci/mmole) and 15% glycerol. At intervals triplicate samples of 100 µl were taken.

OTHER PROCEDURES

Protein concentrations were determined by the method of LOWRY et al. (7), using bovine serum albumine as a standard and DNA concentrations were determined by the method of BURTON (8), using calf thymus DNA as a standard.

RESULTS AND DISCUSSION

The kinetics of DNA synthesis by liver nuclei from control mice and from animals infected for 3 hours with 5 x 10^8 PFU of FV_3 are shown in Fig. 1. As can be seen from the figure, the control and the infected nuclei incorporated [^{3}H] dTTP into acid insoluble material for 30 minutes after incubation and at a

TABLE I :

EFFECT OF FV_3 ON DNA SYNTHESIS BY ISOLATED NUCLEI

The standard reaction mixture contained the following components in a final volume of 125 µl : 0.025 M Tris HCl buffer (pH 8), 0.005 M 2-mercaptoethanol, 0.005 M EDTA, 0.025 M KCl, 0.01 M magnesium acetate, 0.002 M ATP, 0.015 mM each of d ATP, d CTP, d GTP and [^{3}H] d TTP (specific activity 15 Ci/mmole) and 15% glycerol.
When exogenous primer was used, 10 µg of calf thymus DNA was added in each assay tube.
Incubation time was 1 hour and each assay contained 100 µg of nuclear protein.

System	Incorporation of [^{3}H] d TTP pmoles/mg protein	
	Endogenous primer	Exogenous primer
Control I	25.41	68.49
Infected mice for 3 hours	14.49 (57%)	40.53 (59.1%)
Control II	33.9	110.13
Infected mice for 5 hours	10.47 (30.8%)	44.46 (40.3%)

linear rate for the first 10 minutes. In the case of the nuclei from infected animals, the synthesis of DNA was markedly decreased : after 10 and 30 minutes of incubation, the incorporation was 59% and 52% respectively of that of the control animals. Under the conditions of the assay, the incorporation of [^{3}H] dTTP was proportional to the amount of nuclei added up over a range of at least 300 µg of nuclear proteins.

The addition of an exogenous primer DNA stimulated the reaction noticeably which corresponds with the results obtained in other systems (6 - 9).

The data in table I indicate that, if the degree of stimulation obtained by addition of calf thymus DNA was roughly the same with nuclei from infected and non infected animals, the amount of dTTP incorporation per mg protein, however,

TABLE II :

EFFECT OF ADDITION OF VARIOUS SUBSTANCES ON THE DNA SYNTHESIZED BY ISOLATED MICE LIVER NUCLEI

The complete system was the standard reaction mixture described in table I. After 1 hour of incubation at 37°C, the tubes were heated to 65°C for 4 min. to inactivate the enzymes and then cooled. Additions were made as given in the table and incubation continued for 1 hour at 37°C. When TCA was added the tubes were incubated for 10 min. at 90°C.

Experiment	[^{3}H] d TTP incorporated pmoles/mg protein	
	Endogenous primer	Exogenous primer
Control	21	49.29
+ deoxyribonuclease (80 µg/ml	0.39 (1.85%)	0.43 (0.87%)
+ ribonuclease (80 µg/ml)	19.2 (91.40%)	50.7 (102.80%)
+ trichloracetic acid (10%)	0.13 (0.61%)	0.3 (0.60%)

was very much lower in the case of nuclei from infected animals so that the final inhibition was the same as that obtained with endogenous DNA.

Since the addition of DNA to isolated nuclei is comparable to studying a crude solubilized DNA polymerase system (6), this would indicate that in FV_3 infected nuclei there is a lower amount of active enzyme than in nuclei from non infected mice. Thus, an interaction between FV_3 proteins and DNA, even if it occurs *in vitro* (10), would not be the only mechanism responsible for the inhibition of DNA replication or DNA repair *in vivo*.

The data in table II indicate that the product of the reaction shows the properties of DNA. Treatment of the acido-precipitable product with hot TCA or with deoxyribonuclease led to almost complete solubilization of the synthesized product, whereas ribonuclease treatment had no effect. However, it can not

TABLE III :

DEOXYRIBONUCLEASE ACTIVITY IN LIVER NUCLEI FROM CONTROL MICE AND FROM MICE INFECTED WITH 5 x 10^8 PFU OF FV_3 DURING 5 HOURS.

Deoxyribonuclease I (alkaline) was assayed in a reaction mixture described in table I. Each assay tube contained 20 µg of ^{14}C labeled KB cell DNA (9 x 103 cpm/µg of DNA) and 150 µg of the nuclear preparation to be tested. At the end of a 30 min. incubation at 37°C the tubes were chilled 100 µl of a 1 mg per ml solution of calf thymus DNA were added and the DNA was precipitated by addition of 150 µl of 1 N perchloric acid. After standing for 10 min. the tubes were centrifuged and samples of 100 µl of supernatant fluid were removed for scintillation counting.

	Label recovered in the supernatant (cpm)
Control	4,872
Infected mice for 5 hours	3,777

be postulated that the synthesis in our system represents DNA replication rather than DNA repair.

A higher degradation of the product synthesized by the nuclei from the infected animals could have explained the reduction of incorporation into acido-insoluble material. Therefore, the deoxyribonuclease activity of liver nuclei from infected and non infected mice was checked. As shown in table III the deoxyribonuclease activity was not increased in nuclei from infected animals, moreover it was slightly decreased.

CONCLUSIONS

The inhibition of DNA polymerase activity in isolated liver nuclei from mice infected with FV_3 correlates well with the previously described inhibition of thymidine incorporation in the liver of infected mice (3). These results supply further evidence of a very early interaction of FV_3 with the functions

of the nuclei and correspond with the ultrastructural lesions of the hepatocytes nuclei (4). They support the idea that the FV_3 induced hepatitis in mice is related with a perturbation of the macromolecular metabolism starting with nuclear alterations.

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