A C T A B I O C H I M I C A P O L O N I C A

Vol. 36 1989 No. 2

ELŻBIETA ANUSZEWSKA and JADWIGA KOZIOROWSKA

CHARACTERISTICS OF MNNG INDUCED REPAIR SYNTHESIS AND DNA SYNTHESIS INDUCED BY HUMAN CYTOMEGALOVIRUS

Institute for Drug Research and Control,
Chełmska 30/34; 00-725 Warszawa, Poland

Received 28 January, 1987; Revised 19 December, 1988

DNA synthesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in mouse embryo and human embryo cells was compared with DNA synthesis induced in these cells by human cytomegalovirus. In virus infected human embryo cells grown in the medium depleted of arginine DNA synthesis showed resistance to hydroxyurea and arabinofuranosylcytosine, similarly as repair synthesis induced by MNNG. DNA synthesis induced by the virus in mouse embryo cells was partially sensitive to both inhibitors.

Treatment of mammalian cells with various chemical agents results in DNA damage and subsequent DNA repair synthesis. Infection with carcinogenic DNA viruses e.g. polyoma virus or SV40 virus induces cellular DNA synthesis [1, 2]. Both these events appear to be of importance for the initiation of carcinogenesis.

Human cytomegalovirus (HCMV)[1] stimulates host cell DNA synthesis in both permissive and non-permissive cells [3-6]. Increased rate of DNA synthesis by HCMV was demonstrated in cell cultures in which DNA replication had been suppressed prior to infection [7]. The findings of Gönczöl *et al.* [8] indicate that a polypeptide growth factor might be involved in the events leading to the enhancement of cellular DNA synthesis by HCMV both in permissive and non-permissive cells. According to

[1] Abbreviations: MEM, minimal essential medium; ADM, arginine deficient medium; NaCl/P_i, phosphate buffered saline; HCMV, human cytomegalovirus; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DMSO, dimethylsulphoxide; $TCID_{50}$, tissue culture infectious dose; [^{3}H]dThd, [^{3}H]thymidine; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

St. Jeor *et al.* [9] DNA synthesis induced by HCMV infection in both these kinds of cells represents normal semiconservative replication but not repair synthesis. Moreover HCMV can malignantly transform both these kinds of cells [10 - 13].

The aim of the present study was to search for common features of repair DNA synthesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and DNA synthesis induced by HCMV in the absence or presence of hydroxyurea or and 1-*β*-D-arabinofuranosylcytosine (araC). The experiments were performed on non-infected or HCMV infected human embryo cells or mouse embryo cells cultivated in the medium depleted of arginine. Mouse embryo cells are an abortive system for human CMV in which the virus adsorbs, penetrates and produces early antigens without producing any detectable progeny virus [11, 13, 14, 15]. Without arginine in the medium the infectious virus is not produced [16, 17].

MATERIALS AND METHODS

Chemicals. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) from Serva; 1-*β*-D-arabinofuranosylcytosine (araC) from Upjohn; hydroxyurea from Sigma; [^{3}H]dThd (25 Ci/mmol) from Amersham; foetal bovine serum from Gibco.

Cells. Primary and secondary cultures of mouse embryonic cells (ME) from PzH:SFISS mice and human embryonic cell line CLV95 (HE) were used. The latter cells were obtained from Dr. Natalia Mazurowa from Serum and Vaccines Research Laboratories, Warsaw. The cells were checked by the method of Garrett & Reeson [18] to assure that they were free of Mycoplasma contamination.

Virus strain. The strain of CMV used in these studies was Ad-169, obtained from the National Bacteriological Laboratory, Sweden. Virus stocks were prepared in HE cells. Confluent monolayers of HE cells were infected with HCMV and incubated for 2 h at 37°C to allow virus adsorption. Maintenance medium was then added and exchanged at 5-day intervals. Seven to 14 days post-infection, when the cytopathic effect had developed in 90% - 100% of the cells, the cultures were harvested. The total amount of infectious virus was measured after the cells had been disrupted by freezing and thawing. The titre of virus varied over a range of 3.7×10^6 to 10^7 $TCID_{50}$/ml (stock solutions). Virus stock was rapidly frozen and stored at −130°C.

Measurement of repair synthesis. Human or mouse embryo fibroblasts were seeded into 35 mm plastic Petri dishes at a density of 2×10^5 cells/ml (2 ml per dish) and were grown to subconfluence at 37°C in a 5% CO_2 humidified atmosphere for 48 h in MEM supplemented with 10% foetal bovine serum and antibiotics. After 48 h the medium was removed and the

cells were washed with NaCl/P_i, then ADM supplemented with 10% of dialyzed foetal bovine serum was added to cultures and they were placed in the incubator for the next 48 h. After a further 1 h incubation with araC (0.25 mg/ml) and/or hydroxyurea (0.75 mg/ml), MNNG (15 μg/ml) dissolved in DMSO and [^{3}H]dThd (1 μCi/ml) were added and the cultures were incubated for 3 h. Incorporation of [^{3}H]dThd into DNA was stopped by addition of cold thymidine at the concentration of 50 μg/ml and the cells were washed several times with cold NaCl/P_i and dried in air. To determine the incorporation of [^{3}H]dThd into DNA, the cells were treated with 1 M NaOH for 1 h at 37°C to digest RNA. After incubation the suspension was cooled and 10% trichloroacetic acid was added. The cell fractions insoluble in trichloroacetic acid were collected onto millipore filters, dried and placed in vials containing 8.0 ml of a mixture of toluene with POPOP and PPO for subsequent liquid scintillation counting. Controls contained all additions except MNNG. DNA repair synthesis was expressed as percentage of [^{3}H]dThd incorporation as compared with controls taken as 100.

Measurement of DNA synthesis in virus-infected cells in the presence of inhibitors. Cells were routinely grown as described above. The cultures 48 h old were infected with HCMV (1 $TCID_{50}$/cell). After 2 h adsorption the cells were washed with NaCl/P_i and the arginine deficient medium (ADM) was added.

Control cultures and virus-infected cultures were exposed at 48 h post--infection to hydroxyurea (0.75 mg/ml) and/or araC (0.25 mg/ml) for 1 h and then incubated with [^{3}H]thymidine (1 μCi/ml) for another 3 h. The radioactivity incorporated into the trichloroacetic acid-insoluble material was counted as described above.

Determination of virus yield. Human embryonic fibroblast monolayers were inoculated with 1 $TCID_{50}$/cell of virus. After an adsorption period of 1 h the inoculum was removed, the monolayer was washed three times with NaCl/P_i then covered with medium. The end point of infectivity was read 14 days after infection. The cells were thoroughly washed with NaCl/P_i trypsinized, resuspended, and made up to the original volume with medium. The suspension was sonicated and centrifuged. The supernatant was titrated in tube cultures of human fibroblasts. The results were calculated according to the Reed-Muench method.

RESULTS

The results presenting the inhibitory effect of hydroxyurea and/or araC on the synthesis of DNA in mouse and human embryo cells are summarized in Table 1. In the presence of both inhibitors the residual semiconservative

Table 1

Inhibitory effect of hydroxyurea and araC on the level of semiconservative DNA synthesis in cultured human embryo (HE) and mouse embryo (ME) cells

The experiments were carried out as described in the Methods. The results represent the mean of three determinations with S.E. < 20%

Kind of medium	HE cells CLV95		ME cells Pzh:SFISS	
	c.p.m./culture	%	c.p.m./culture	%
MEM	11 968	100.0	9 735	100.0
MEM + hydroxyurea	789	6.6	871	8.9
MEM + araC	1 039	8.7	487	5.0
MEM + araC + hydroxyurea	697	5.8	152	1.5
ADM	2 990	24.9	2 564	26.3
ADM + hydroxyurea	595	4.9	504	5.2
ADM + araC	388	3.2	212	2.1
ADM + araC + hydroxyurea	200	1.61	99	1.0

DNA synthesis was less than 2% of control value. Effects of MNNG on [^{3}H]thymidine incorporation into human embryo cells are presented in Fig. 1. The incorporation of [^{3}H]dThd into DNA was the greatest in the cells grown in ADM in the presence of both hydroxyurea and araC. A similar result was obtained in the experiments with mouse embryo cells (not shown).

Figure 2 shows that in human embryo cells grown in ADM 48 h after infection the incorporation of [^{3}H]thymidine into DNA of HCMV infected cells was about 6 times higher than in control cells. On addition of hydroxyurea the incorporation of [^{3}H]dThd was half that in the infected cultures grown without any inhibitor; however, in the presence of both

Table 2

HCMV yield human embryo (HE) and mouse embryo (ME) cells 48 h post infection

Tube cultures of human embryo (HE) or mouse embryo (ME) cells were infected with HCMV and maintained on either MEM or ADM for 48 h. After this time the yield of titratable virus was determined

Cells	Medium	$TCID_{50}$/0.1 ml
HE	MEM	$10^{5.6}$
HE	ADM	$10^{3.8}$
ME	MEM	$10^{2.7}$
ME	ADM	$10^{2.2}$

inhibitors together or araC alone it was very similar to that in the infected cultures without inhibitor.

From Figure 3 it is evident that in mouse embryo cells, grown in the absence of arginine, similarly as in human embryo cells, HCMV infection stimulated DNA synthesis. However, both hydroxyurea and araC reduced

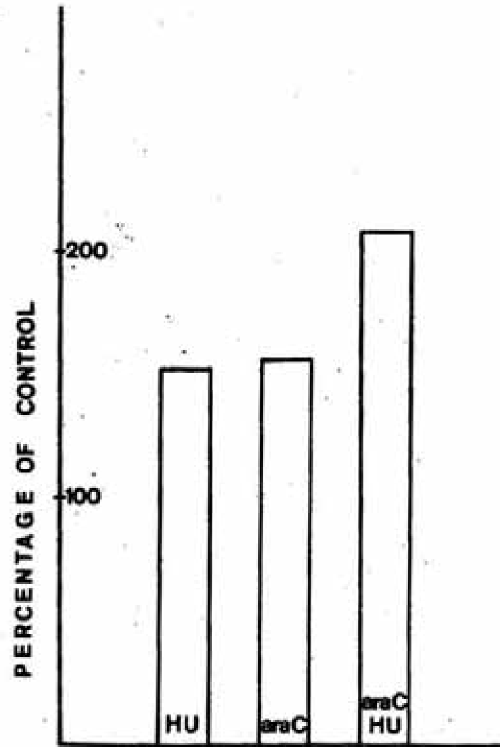

Fig. 1. Effect of MNNG treatment on [^{3}H]thymidine incorporation in human embryo cells in the presence of hydroxyurea and/or araC. Human embryo cells were grown in arginine deficient medium as described in Methods. The results of the experiments are expressed as the respective percentages of controls untreated with MNNG but treated with hydroxyurea and/or araC taken as 100. The results represent the mean of three determinations with S.E. $<20\%$.

DNA synthesis in these cells. The extent of this inhibition was lower (55, 32 and 14% respectively, as compared with infected cells) than in the uninfected cells (Table 1).

The effect of lack of arginine on the extent of virus infectivity in permissive human embryo cells and mouse embryo cells is presented in Table 2. The amounts of infectious virus recovered from human embryo

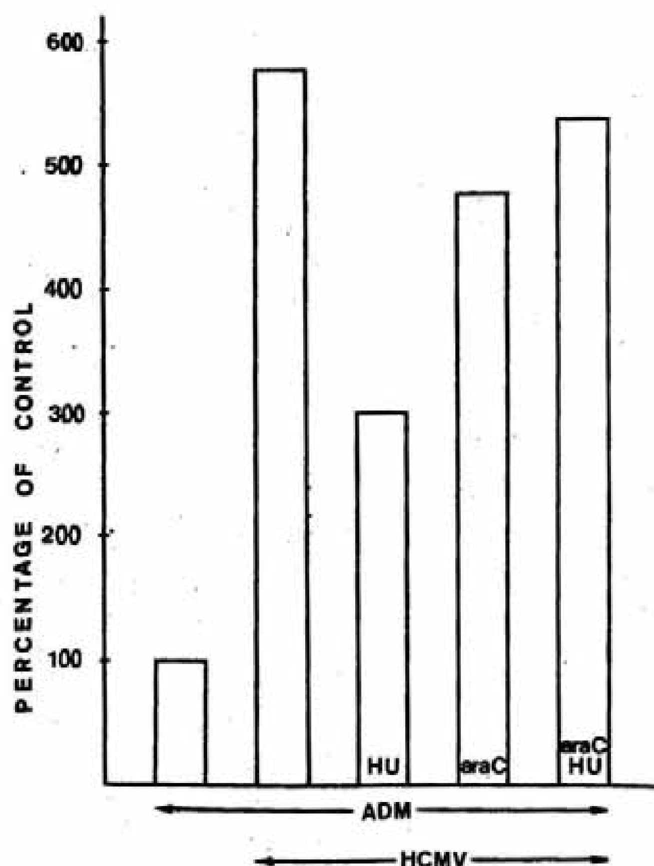

Fig. 2. Effects of inhibitors on DNA synthesis in human embryo cells infected with HCMV. Subconfluent 48 h old cultures were infected with HCMV (1 $TCID_{50}$ cell) and incubated for 48 h in the arginine deficient medium. The results of the experiments are expressed as percentage of controls (uninfected cultures grown in ADM). Data are the mean of three determinations with S.E. $<20\%$

cells grown in MEM were similar to input levels while the amounts of the virus recovered from human embryo cells grown in ADM or mouse embryo cells cultivated in both kinds of media were lower than input levels.

DISCUSSION

It has been established that HCMV-induced increased DNA synthesis in confluent cultures of permissive cells can be demonstrated in those cells in which DNA synthesis has been suppressed prior to infection [7, 9]. However, in subconfluent cultures HCMV did not stimulate synthesis

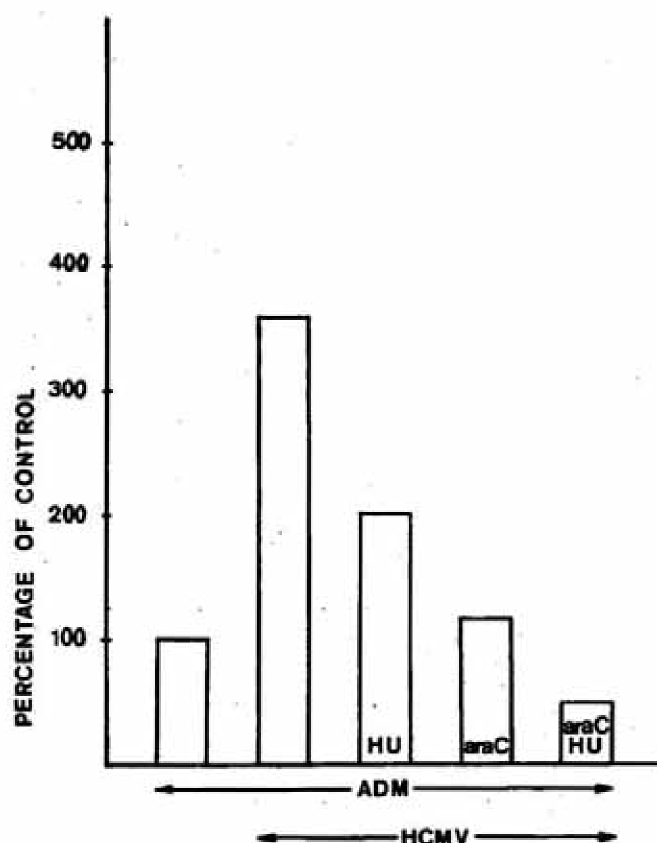

Fig. 3. Effects of inhibitors on DNA synthesis in mouse embryo cells infected with HCMV. The procedure applied is described in Materials and Methods. The results of the experiments are expressed as percentages of controls (uninfected cultures grown in ADM). Data are the mean of three determination with S.E. $<20\%$

of cellular DNA because those cells are more susceptible to productive infection than are cells in confluent cultures [7]. In our study we have demonstrated that HCMV is capable of inducing DNA synthesis in subconfluent cultures of permissive cells in conditions in which background level of DNA synthesis is lowered by arginine starvation. These findings gave us the opportunity to assess changes in the synthesis of DNA caused under the experimental conditions used for the detection of MNNG induced unscheduled DNA synthesis.

From the results of the experiments it was possible to conclude that DNA synthesis in the infected human embryo cells at 48 h post-infection was resistant to araC and to araC together with hydroxyurea, thus bearing the same characteristics as the MNNG induced repair synthesis.

AraC is an inhibitor of DNA polymerases [19, 20]. Hydroxyurea affects DNA synthesis by lowering intracellular pools of deoxyribonucleotides through inhibition of ribonucleotide reductase [21]. Hirai *et al.* [5] suggested that in permissive cells grown in standard media HCMV induce a novel polymerase before the induction of cellular DNA and before the reproduction of viral DNA. This polymerase seems to be involved in the reproduction of viral DNA and may be induced even in the presence of araC. In arginine depleted media HCMV genomes seem to persist [16, 22]. It is therefore possible that the observed resistance to araC of the induced DNA synthesis in human embryo cells is also related to the appearance of a novel polymerase. Since in the same cells MNNG induced unscheduled DNA synthesis that takes place in the presence of araC, it seems possible that a polymerase bearing the same characteristics as the polymerase induced by HCMV takes part in this synthesis.

The results of this study show also that DNA synthesis in the infected mouse embryo cells is less sensitive to araC than in uninfected control cells. In nonpermissive cells HCMV seems to stimulate cellular polymerase [6]. Further studies are needed to explain the possible relationship between this event and the changes observed now in the response of DNA synthesis to the action of araC.

As it is well known from numerous studies, chemical agents may enhance viral carcinogenesis and viral agents have a stimulatory effect on chemical carcinogenesis. Our study provides a model system for further characterization of similarities and differences between the characteristics of DNA synthesis induced by a virus and a chemical agent. There is considerable evidence that pretreatment of cells with DNA-damaging agents can enhance the reactivation of carcinogen-pretreated viruses, including herpes simplex, SV40, and parvovirus [23 - 25]. Presumably these effects lead to induction of DNA repair enzymes although the putative genes and proteins involved in these processes have not been identified.

It would be of interest to determine in further studies whether the enzymes involved in repair processes induced by MNNG are involved in the reactivation of HCMV damaged DNA, and to explain the biological significance of virus-induced enzymes in the repair of cellular DNA.

We express thanks to Mrs Katarzyna Adamska-Kłos and Mrs Mieczysława Subocz for excellent technical assistance.

REFERENCES

1. Dulbecco, R. I., Hartwell, H. & Vogt, M. (1965) Induction of cellular DNA synthesis by polyoma virus. *Proc. Natl. Acad. Sci. U.S.A.*, **53**, 403-410.
2. Gershon, D., Sachs, L. & Winocour, E. (1966) The induction of cellular DNA synthesis

by simian virus 40 in contact-inhibited and in X-irradiated cells. *Proc. Natl. Acad. Sci. U.S.A.*, **56**, 918-925.

3. Furukava, T., Tanaka, S. & Plotkin, S. A. (1975) Stimulation of macromolecular synthesis in guinea pig cells by human CMV. *Proc. Soc. Exp. Biol. Med.*, **148**, 211-214.
4. Albrecht, T. B., Nachtigal, M., St. Jeor, S. C. & Rapp, F. (1976) Induction of cellular DNA synthesis and increased mitotic activity in syrian hamster embryo cells abortively infected with human cytomegalovirus. *J. Gen. Virol.*, **30**, 167-177.
5. Hirai, K., Furukawa, T. & Plotkin, S. A. (1976) Induction of DNA polymerase in WI-38 guinea pig cells infected with human cytomegalovirus (HCMV). *Virology*, **70**, 251-255.
6. Kamiya, S., Tanaka, J., Ogura, T., Sato, M. & Hatano, M. (1986) Rabbit kidney cells abortively infected with human cytomegalovirus are arrested in mitotic phase. *Arch. Virol.*, **89**, 131-144.
7. DeMarchi, J. M. & Kaplan, A. S. (1977) The role of defective cytomegalovirus particles in the induction of host cell DNA synthesis. *Virology*, **82**, 93-99.
8. Gönczöl, E. & Plotkin, S. A. (1984) Cells infected with human cytomegalovirus release a factor that stimulates cell DNA synthesis. *J. Gen. Virol.*, **65**, 1833-1837.
9. St. Jeor, S. C., Albrecht, T. B., Funk, F. D. & Rapp, F. (1974) Stimulation of cellular DNA synthesis by human cytomegalovirus. *J. Virol.*, **13**, 353-362.
10. Albrecht, T. B. & Rapp, F. (1973) Malignant transformation of hamster embryo fibroblast following exposure to ultraviolet-irradiated human cytomegalovirus. *Virology*, **55**, 53-61.
11. Boldogh, I., Gönczol, E. & Vaczi, L. (1978) Transformation of hamster embryonic fibroblast cells by UV-irradiated human cytomegalovirus. *Acta Microbiol. Acad. Sci. Hung.*, **25**, 269-275.
12. Geder, L. R., Lausch, R., O'Neill, F. & Rapp, F. (1977) Oncogenic transformation of human embryo lung cells by human cytomegalovirus. *Nature (London)*, **265**, 184-186.
13. Fioretti, A., Furukawa, T., Santoli, D. & Plotkin, S. A. (1973) Nonproductive infection of guinea pig cells with human cytomegalovirus. *J. Virol.*, **11**, 998-1003.
14. Gönczol, E., Boldogh, I. & Vaczi, L. (1975) Virus-specific changes in mouse cells inoculated with a strain of cytomegalovirus. *Acta Microbiol. Acad. Sci. Hung.*, **22**, 275-283.
15. Waner, I. L. & Weller, T. H. (1974) Behavior of human cytomegalovirus in cell cultures of bovine and simian origin. *Proc. Soc. Exp. Biol. Med.*, **145**, 379-384.
16. Garnett, H. (1975) The effect of arginine deprivation on the cytopathogenic effect and replication of human cytomegalovirus. *Arch. Virol.*, **48**, 131-145.
17. Gönczöl, E., Boldogh, I. & Vaczi, L. (1975) Effect of arginine deficiency on the reproduction of human cytomegalovirus. *Acta Microbiol. Acad. Sci. Hung.*, **22**, 263-270.
18. Garrett, A. J. & Reeson, D. E. (1975) Rapid screening of tissue culture cells for mycoplasma contaminations. *J. Biol. Scand.*, **3**, 181-184.
19. Dunn, W. C. & Reagan, J. D. (1979) Inhibition of DNA excision repair in human cells by arabinofuranosyl cytosine: Effect on normal and xeroderma pigmentosum cells. *Mol. Pharmacol.*, **15**, 367-374.
20. Yoshida, S., Yamada, M. & Masaki, S. (1977) Inhibition of DNA polymerase-α and -β of calf thymus by 1-β-D-arabinofuranosylcytosine-5′-triphosphate. *Biochim. Biophys. Acta*, **477**, 144-150.
21. Timson, J. (1975) Hydroxyurea. *Mutation Res.*, **32**, 115-132.
22. Aono, J. & Minamishima, Y. (1984) Effect of arginine deprivation on murine cytomegalovirus replication. *Microbiol. Immunol.*, **28**, 129-133.
23. DasGupta, U. B. & Summers, W. C. (1978) Ultraviolet reactivation of herpes simplex virus is mutagenic and inducible in mammalian cells. *Proc. Natl. Acad. Sci. USA*, **75**, 2378-2381.
24. Sarasin, A., Bourre, F. & Benoit, A. (1982) Error prone replication of ultraviolet-

-irradiated simian virus 40 in carcinogen treated monkey kidney cells. *Biochimie*, **64**, 815-821.

25. Cornelis, J. J., Su, Z. Z. & Rommelaire, J. (1982) Direct and indirect effect of ultraviolet light on the mutagenesis of parvovirus M-1 in human cells. *EMBO J.*, **1**, 693-699.