THE EFFECT OF METHYLGLYOXAL BIS(GUANYLHYDRAZONE) ON VACCINIA VIRUS REPLICATION

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SUMMARY: *Methylglyoxal bis(guanylhydrazone), an inhibitor of S-adenosyl-*L-methionine decarboxylase, inhibits partially the yield of infectious, progeny virus in vaccinia-infected HeLa cells. This effect is reversed by the simultaneous addition of spermidine showing that this polyamine is required for optimal virus growth. Although DNA, RNA and protein synthesis are unaffected, the formation of DNA-containing, cytoplasmic inclusions characteristic of vaccinia infection occurred at a reduced frequency compared with non-inhibited controls. Thus, the association of virus DNA with cytoplasmic inclusions is decreased in the absence of spermidine synthesis. The nature of residual virus replication in the presence of the inhibitor is discussed.

Previous studies in this laboratory have shown that infection of HeLa cells with vaccinia virus results in both quantitative and qualitative changes in ornithine decarboxylase (EC. 4.1.1.17) activity (1). This enzyme catalyzes the formation of putrescine, the initial step in polyamine biosynthesis. Two polyamines, spermidine and spermine, have been shown to be present in vaccinia virions and the synthesis of these polyamines continued after infection (2). The biosynthesis of spermidine and spermine in mammalian cells is affected by *S-adenosylmethionine decarboxylase* (EC 4.1.1.50). Methylglyoxal bis(guanyl-hydrazone) (MGBG) is a potent inhibitor of this enzyme (3). This paper investigates the effect of MGBG on vaccinia virus replication and describes the partial inhibition of the production of infectious, progeny virus. This decreased yield is accompanied by a reduction in the frequency of formation of DNA-containing inclusions in the cytoplasm of vaccinia-infected cells.

METHODS:

The laboratory line of HeLa cells used (4) was grown in Eagle's minimum essential medium (MEM) containing 5% calf serum. Confluent monolayers of HeLa

cells in test-tubes (1 to 2 x 10^6 cells/tube) were infected with the Lister strain of vaccinia virus using 5 plaque-forming units (p.f.u.)/cell. After adsorption for one hr the inocula were removed, the monolayers washed with Hanks' balanced salt solution and the infected cells maintained subsequently in medium containing various concentrations of the inhibitor. The maintenance medium used was MEM supplemented with 2% calf serum. All procedures were carried out at 37°C. After incubation for 18 hrs the infected cells were disrupted by two cycles of freezing and thawing followed by ultrasonic treatment. Infectivity titres were determined by plaque formation in cultures of Vero cells.

The synthesis of DNA, RNA and protein in infected and control cell cultures was examined by incorporation of [^{3}H]-thymidine (sp.act. 5 Ci/mmol), [^{14}C]-uridine (sp.act. 492 mCi/mmol) and [^{3}H]-leucine (sp.act. 57 Ci/mmol), respectively. All labelled compounds were obtained from The Radiochemical Centre, Amersham, Buckinghamshire. Infected and sham-infected HeLa cell monolayers were maintained in medium containing the appropriate labelled precursor together with a suitable concentration of the inhibitor. After 6 hrs the amount of incorporated radioactivity was determined (4). The incorporation of [^{3}H]-thymidine into the cytoplasmic fraction of infected cells was measured as described previously (5).

The appearance of cytoplasmic, DNA-containing inclusions in vaccinia virus-infected cells was visualized by acridine orange staining (6).

Methylglyoxal bis(guanylhydrazone) dihydrochloride monohydrate was obtained from Aldrich Chemical Co. and spermidine trihydrochloride from Sigma Chemical Co.

RESULTS:

The production of infectious, progeny virus in vaccinia-infected HeLa cells was reduced progressively in the presence of concentrations greater than 0.1 mM-MGBG. Maximal inhibition was observed with 0.5 mM-MGBG and this concentration of the inhibitor resulted in a 70% reduction of virus yield (Table 1). The specificity of this effect was examined by the addition of 1.0 mM-spermidine to maintenance medium containing 0.5 mM-MGBG supplied to HeLa cell cultures immediately after infection. Complete reversal of the inhibitory effect was obtained (Table 1). These results show that spermidine is required for production of the full yield of infectious, progeny virus and that synthesis of this polyamine continues in vaccinia-infected cells.

Since higher concentrations of MGBG did not effect further reductions of virus yield and similar results were obtained using a serum-free, modified Eagle's medium, residual virus replication may have resulted from the utilization of spermidine present in intracellular pools at the time of infection.

Table 1. The effect of MGBG on the production of infectious vaccinia virus in HeLa cell monolayers

MGBG (mM)	Infectivity titre (p.f.u./ml)	Virus yield (%)
0	1.10×10^7	100
0.10	1.12×10^7	100
0.25	7.10×10^6	65
0.50	3.62×10^6	32
1.00	3.83×10^6	34
0.50 + 1.00 mM spermidine	9.95×10^6	91

Consequently, confluent monolayers of HeLa cells were maintained in the presence of 0.5 mM-MGBG for 24 hrs, then infected and maintained subsequently in the presence of the same concentration of the inhibitor. Titration of virus released from these cells at 18 hrs after infection showed no significant difference in yield compared with virus released from cells exposed to the inhibitor from the time of infection only. The infectivity titre of virus from cells maintained in the presence of MGBG both before and after infection was 2.74×10^6 p.f.u./ml and that from cells treated with the inhibitor after infection was 2.85×10^6 p.f.u./ml. Microscopic examination of uninfected cultures maintained for 42 hrs in medium containing 0.5 mM-MGBG did not reveal any cytotoxic effects.

The replication of vaccinia virus occurs in the cytoplasm of the infected cell. Consequently, incorporation of [^{3}H]-thymidine into the cytoplasmic fraction of vaccinia-infected cells is a measure of virus-specific DNA synthesis which, in the system used, is complete by 6 hrs post-infection (4). In the presence of 0.5 mM-MGBG such incorporation was unaffected. Under the same conditions the incorporation of thymidine into whole, uninfected cells was similar to control cells. Neither the incorporation of [^{14}C]-uridine nor [^{3}H]-leucine into infected or control cells was affected by the inhibitor

Table 2. The effect of MGBG on macromolecular synthesis in uninfected and vaccinia virus-infected HeLa cells

	Incorporated radioactivity (dpm x 10^{-3})					
	Uninfected			Infected		
Precursor	Control	Inhibited	% control	Control	Inhibited	% control
[^{3}H]-thymidine	6,559	6,558	100	534*	550*	103
[^{14}C]-uridine	940	914	97	615	653	106
[^{3}H]-leucine	633	679	107	676	685	101

* Radioactivity incorporated into cytoplasmic fractions only

(Table 2). These results show that a concentration of MGBG which produces maximum inhibition of virus growth is without measurable effect on DNA, RNA or protein synthesis in either uninfected or vaccinia-infected HeLa cells.

The synthesis of vaccinia virus DNA is accompanied normally by the formation of cytoplasmic, DNA-containing inclusions. These inclusions can be visualized by acridine orange staining of vaccinia-infected cells. In the presence of 0.5 mM-MGBG cytoplasmic inclusions were observed when infected cultures were examined at 6 hrs post-infection confirming that virus DNA is synthesized under these conditions. Cytoplasmic inclusions were not distinguishable in every control, infected cell because the HeLa cell nucleus occupies a large proportion of the total cell volume. However, their frequency in MGBG-treated cells was about 40% of that in non-inhibited cultures (Table 3). The inclusions in both inhibited and control cells subsequently became more diffuse in appearance, a stage of development associated with the maturation of virus particles in unstained cultures (7). Concentrations less than 0.1 mM-MGBG were without effect on the frequency of cytoplasmic inclusions. These results show that the association of virus DNA with cytoplasmic inclusions is

Table 3. The effect of canaline on the frequency of DNA-containing, cytoplasmic inclusions in vaccinia virus-infected HeLa cells

Treatment	Number of cells examined	Number of cells showing inclusions	Cells showing inclusions(%)
Without MGBG	572	207	36.2
With 0.5 mM-MGBG	631	90	14.3

reduced markedly in the presence of concentrations of the inhibitor that affect the yield of infectious, progeny virus.

DISCUSSION:

Polyamines have been shown to be constituent parts of virions representing two groups of large, DNA-containing animal viruses. Exogenous ornithine could function as a precursor to the polyamines present in herpes virions only when available before infection (8). Other studies showed that the synthesis of spermidine and spermine from putrescine is inhibited in herpes simplex virus-infected cells and, consequently, MGBG does not affect virus replication (9). However, ornithine decarboxylase activity increases in vaccinia-infected cells (1) and ornithine can serve as a precursor in the synthesis of those polyamines found in vaccinia virions when added before or after infection of cells with virus (2). The present study shows that MGBG reduces markedly the production of infectious, progeny virus in vaccinia-infected cells. This reduced yield results from the effect of MGBG on polyamine synthesis since the inhibitory effect is reversed by the simultaneous addition of exogenous spermidine. These results show that spermidine is required for the replication of vaccinia virus and confirm previous observations that polyamine biosynthesis continues in vaccinia-infected cells.

Increased RNA and protein synthesis resulting from concanavalin A-induced transformation of lymphocytes is accompanied by marked elevation of cellular polyamine levels (10). These increases in macromolecular synthesis continue

unaltered following inhibition by MGBG of spermidine and spermine synthesis. Under these conditions, however, there was a 60% reduction in the incorporation of thymidine into DNA and in the rate of entry of cells into mitosis (11). In the presence of a concentration of MGBG that reduces maximally the production of infectious, progeny virus there was no measurable effect on DNA, RNA or protein synthesis in either uninfected or vaccinia-infected HeLa cells. Although the incorporation of thymidine was unaffected, the formation of DNA-containing inclusions in the cytoplasm of infected cells showed a 60% reduction in frequency compared with untreated controls. Inhibition of spermidine synthesis, therefore, results in a marked suppression of the association of virus DNA with cytoplasmic inclusions. This polyamine has been shown to stabilize the condensed DNA conformations in isolated nucleoids from bacterial cells (12). The parallel reductions in inclusion formation and production of progeny virus in the presence of MGBG suggest a similar role in vaccinia virus replication. Indeed, spermidine has been shown to be present in vaccinia virus cores (2).

Since higher concentrations of MGBG did not effect further reductions of virus growth and similar virus yields were obtained following exposure of cells to the inhibitor both before and after infection, it is unlikely that residual virus replication results from utilization of intracellular pools available at the time of infection. Although spermidine synthesis is affected, accumulation of putrescine continues in the presence of the inhibitor (10). Thus, residual virus growth in the presence of MGBG suggests the possible participation of putrescine in vaccinia virus replication. The proliferation of rat hepatoma cells is blocked following inhibition of ornithine decarboxylase activity by α-methyl ornithine. Although putrescine concentrations fell rapidly there was no inhibition of DNA synthesis until spermidine levels dropped significantly (13). The replication of vaccinia virus in HeLa cells is inhibited by canaline, an oxyamino analogue of ornithine. Although virus-specific DNA synthesis was reduced by 40% only (5),

the formation of DNA-containing, cytoplasmic inclusions showed a 60% reduction compared with non-inhibited, infected cultures (6). Canaline is a potent inhibitor of pyridoxal phosphate-dependent enzymes, including ornithine decarboxylase (14). These effects of canaline, together with the results of the present study, suggest that the association of virus DNA with cytoplasmic inclusions observed in the presence of the inhibitors can occur without utilization of ornithine for polyamine biosynthesis. The nature of resistance to MGBG that leads to production of infectious, progeny virus is currently under investigation.

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