Human Cytomegalovirus-Induced DNA Polymerase and Its Interaction with the Triphosphates of 1-(2'-Deoxy-2'-Fluoro- β -D-Arabinofuranosyl)-5-Methyluracil, -5-Iodocytosine, and -5-Methylcytosine

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Human cytomegalovirus-induced DNA polymerase and cellular DNA polymerase α were purified by successive chromatography on DEAE-cellulose, phosphocellulose, heparin agarose, and single-stranded DNA agarose columns. The purified virus-induced DNA polymerase was resolved to consist of two polypeptides corresponding to molecular weights of 140,000 and 58,000, as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Virus-induced DNA polymerase and cellular α polymerase were examined for their sensitivities to the triphosphates of 1-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl)-5-methyluracil (FMAUTP), -5-iodocytosine (FIACTP), and -5-methylcytosine (FMACTP). The inhibitive effects of these triphosphates on the DNA polymerases were competitive with regard to the natural substrates; thus FMAUTP competes with dTTP, and FIACTP and FMACTP compete with dCTP. The inhibition constants (K_i) for FMAUTP, FIACTP, and FMACTP of virus-induced DNA polymerase are 0.06, 0.30, and 0.47 μ M, respectively. Cellular DNA polymerase α is much less sensitive to these inhibitors, and its K_i values for FMAUTP, FIACTP, and FMACTP are 0.45, 3.10, and 2.90 μ M, respectively. In addition, human cytomegalovirus-induced DNA polymerase, but not cellular DNA polymerase α , can utilize these analog triphosphates as alternate substrates for their corresponding natural deoxyribonucleoside triphosphates in *in vitro* DNA synthesis.

Many synthetic nucleoside analogs have been proven to be highly potent and selective against herpes simplex virus (HSV) infections. The selectivity appears to be due to the ability of the HSV-induced thymidine kinase (TK) to phosphorylate preferentially the nucleoside analogs to form the key monophosphorylated derivatives in infected cells (9, 10, 17, 18). In addition, the potency of an antiherpes nucleoside analog has been correlated with its ability to be differentially phosphorylated by virus-encoded TK *in vitro* (3, 5, 6). This selective alternative substrate theorem explicably fits all the existing antiherpes nucleoside analogs (7, 11, 24) because TK⁻ mutants of HSV are essentially unaffected by this category of compounds (7, 9, 11, 17, 18, 24). Following conversion of monophosphated nucleoside analogs to their triphosphates by host cellular kinases, the triphosphate derivatives became preferential substrates for herpesvirus-specific DNA polymerases and were consequently incorporated into viral DNA (1, 13, 14, 19).

No evidence has been found that a human cytomegalovirus (HCMV)-specific TK activity is present in virus-infected cells (12, 27). Nevertheless, in previous studies (8, 20) it has been demonstrated that certain nucleoside analogs, such as 1-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl)-5-methyluracil (FMAU), -5-iodouracil (FIAU), -5-methylcytosine (FMAC), and -5-iodocytosine (FIAC) were highly effective against HCMV multiplication by virtue of inhibiting viral DNA replication and viral protein synthesis in infected cell cultures (20). To aid in establishing their antiviral mechanism(s) of action, the triphosphates of FMAU, FMAC, and FIAC were prepared and tested against highly purified HCMV-

induced DNA polymerase and cellular DNA polymerase α . The results presented here demonstrate that the triphosphate of each analog could be incorporated into DNA catalyzed preferentially by the virus-induced DNA polymerase, and each triphosphated derivative has more inhibitory effect on the virus-specific DNA polymerase than on cellular DNA polymerase α .

MATERIALS AND METHODS

Chemicals and radiochemicals. The nucleoside analogs (FMAU, FMAC, and FIAC) were provided by J. Fox of the Sloan-Kettering Institute for Cancer Research, Rye, N.Y. The triphosphates of FMAU, FMAC, and FIAC (FMAUTP, FMACTP, and FIACTP, respectively) were prepared as described previously (22). The nonradioactive deoxynucleoside triphosphates dATP, dCTP, dGTP, dTTP, and calf thymus DNA were purchased from Sigma Chemical Co., St. Louis, Mo. [³H]dCTP (30 Ci/mmol) and [³H]dTTP (18 Ci/mmol) were obtained from ICN, Pharmaceuticals, Inc., Chemical Radioisotope Div., Irvine, Calif. Activated DNA was prepared by treating calf thymus DNA with pancreatic DNase at room temperature for 20 min by the procedure of Baril et al. (2). DEAE-cellulose (DE-52), phosphocellulose (P-11), and glass filter paper (GF/C) were from Whatman, Inc., Clifton, N.J. Heparin agarose and single-stranded DNA agarose were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md.

Cells and virus. WI-38 human fibroblasts (Hayflick) were obtained from the American Type Culture Collection, Rockville, Md. Cells at passage 22 to 26 were used. The HCMV Towne strain at passage 36 to 38 was used in this study.

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Enzyme purification. The HCMV-induced DNA polymerase and cellular DNA polymerase α were prepared from 15 confluent Wheaton glass roller bottles each of virus-infected and noninfected WI-38 cells, respectively. The virus-infected cells were harvested 72 h after infection, when more than 80% of cells showed cytopathic effect. Noninfected cells were obtained from a 70% confluent log-phase culture. The procedure of Huang (15) was adapted to the separation and purification of α polymerase and virus-induced DNA polymerase on DEAE-cellulose and phosphocellulose chromatography. The DNA polymerase fractions obtained from the phosphocellulose column were adjusted to 200 μ g/ml in bovine serum albumin (BSA), dialyzed against buffer A (10 mM KPO_4 [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol), and applied to a 15-ml heparin agarose column which had been prewashed with BSA in the same buffer. The column was eluted with a linear 0.1 to 0.7 M KCl gradient; the virus-induced DNA polymerase eluted at 0.32 M, and α enzyme eluted at 0.21 M KCl. These polymerases were subjected to further purification on single-stranded DNA agarose (15 ml) chromatography in a manner identical to that for heparin agarose column chromatography. On the single-stranded DNA agarose column, the virus-induced DNA polymerase eluted at 0.21 M, and α enzyme eluted at 0.11 M KCl. Fractions containing polymerase activity were dialyzed against buffer A (10 mM KPO_4 [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol, 60% glycerol) and stored at -20°C . Proteins were quantitated with a Bio-Rad protein assay kit.

DNA polymerase assays. The polymerase assay system in this study was similar to that used by Huang (15). The viral DNA polymerase reaction mixture contained 60 mM $(\text{NH}_4)_2\text{SO}_4$; 10 mM MgCl_2 ; 0.4 mM dithiothreitol; 20 mM Tris hydrochloride (pH 8.0); 20 μ g of BSA; 20 μ g of activated calf thymus DNA; 100 μ M each of dATP, dCTP, and dGTP; 5 μ M $[^3\text{H}]\text{dTTP}$ (4,000 cpm/pmol); and 20 μ l of a given HCMV-induced DNA polymerase fraction in a total volume of 100 μ l. The reaction mixtures were incubated at 37°C for 30 min. After incubation, acid-insoluble material was collected by precipitation of 50 μ l of the assay mixture onto GF/C filters with 10 ml of 5% trichloroacetic acid containing 2% tetrasodium PP_i per filter. Filters were washed three times (5 min per wash) with cold trichloroacetic acid-pyrophosphate solution, rinsed once with 95% ethanol, heat dried, and counted in liquid scintillation fluid. For α polymerase, the reaction mixtures contained the same ingredients as described above for viral DNA polymerase except that $(\text{NH}_4)_2\text{SO}_4$ was omitted. One unit of DNA polymerase was defined as the amount of enzyme catalyzing the incorporation of 1 pmol of dTMP per min at 37°C under the standard assay conditions. The specific activities of the purified virus-induced DNA polymerase and α polymerase in this study were 5×10^2 and 1.2×10^3 U/ μ g, respectively.

Determination of the kinetic constants K_m and K_i . With FIACPTP as the inhibitor the reaction mixture for assaying HCMV-induced DNA polymerase activity contained 20 mM Tris hydrochloride (pH 8.0); 10 mM MgCl_2 ; 0.4 mM dithiothreitol; 20 μ g of bovine serum albumin; 60 mM $(\text{NH}_4)_2\text{SO}_4$; 20 μ g of activated calf thymus DNA; 10 μ M each of dATP, dTTP, and dGTP; five different concentrations of $[^3\text{H}]\text{dCTP}$ (30 Ci/mmol; 0.33, 0.5, 0.66, 1, and 2 μ M); 0.5 U of enzyme; and three different inhibitor concentrations (0, 1, and 3 μ M). The rate of $[^3\text{H}]\text{dCTP}$ incorporation in the absence of the inhibitor and at different concentrations of dCTP was determined, and the data obtained were used to compute the K_m value by drawing classical Lineweaver-Burk plots (see Fig. 3). The K_i value for FIACPTP was estimated by replotting the

slopes (see inset in Fig. 3). An identical assay condition was employed to determine the K_i value for FMACTP. Similar approaches were used to study the K_i value for FMAUTP against viral DNA polymerase, except that $[^3\text{H}]\text{dTTP}$ (18 Ci/mmol) and dCTP were used to replace $[^3\text{H}]\text{dCTP}$ and dTTP, respectively. The determination of K_m and K_i values in this study was based on the equation for competitive inhibition kinetics:

$$\frac{1}{V} = \left(\frac{K_m}{V_{\max}} \right) \left(1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{\max}}$$

For DNA polymerase α in the presence of FMAUTP inhibitor, the assay mixture consisted of 20 mM Tris hydrochloride (pH 8.0); 10 mM MgCl_2 ; 0.4 mM dithiothreitol; 20 μ g of bovine serum albumin; 20 μ g of activated calf thymus DNA; 100 μ M each of dATP, dCTP, and dGTP; three concentrations of $[^3\text{H}]\text{dTTP}$ (18 Ci/mmol; 5, 10, and 20 μ M); 0.5 U of enzyme; and three different inhibitor concentrations (0, 5, and 10 μ M). When FMACTP and FIACPTP were used as the inhibitors $[^3\text{H}]\text{dCTP}$ and dTTP were added to replace $[^3\text{H}]\text{dTTP}$ and dCTP, respectively, in the assay mixture.

RESULTS

Purification of HCMV-induced DNA polymerase and cellular DNA polymerase α . HCMV-induced DNA polymerase was separable from DNA polymerase α on phosphocellulose chromatography by using a shallow gradient of 0.1 to 0.5 M NaCl (15). The distinction between virus-induced DNA polymerase and α polymerase was ascertained by the addition of 60 mM $(\text{NH}_4)_2\text{SO}_4$ to the reaction mixtures; the virus-induced DNA polymerase activity was enhanced approximately threefold, and α polymerase activity was suppressed. Successive chromatography of the DNA polymerase on heparin agarose column and single-stranded DNA agarose column resulted in 2,500- to 3,000-fold further purification. The elution profiles of virus-specific DNA polymerase and α enzyme were distinguishable; DNA polymerase α and virus-induced DNA polymerase eluted at 0.21 and 0.32 M KCl, respectively, in the heparin agarose column and eluted at 0.11 and 0.22 M KCl, respectively, in the single-stranded DNA agarose column (Fig. 1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified HCMV-induced DNA polymerase showed two protein bands corresponding to molecular weights of 140,000 and 58,000 (Fig. 2).

Kinetics of FMAUTP, FMACTP, and FIACPTP inhibition of DNA polymerases. The abilities of FMAUTP, FMACTP, and FIACPTP to inhibit the utilization of their respective normal triphosphate substrates are presented as the apparent inhibition constant (K_i). Figure 3 illustrates the nature of FIACPTP inhibition on the HCMV-induced DNA polymerase by determining the extent of inhibition with increasing concentrations of the substrate. In assays with activated calf thymus DNA as the template primer, $[^3\text{H}]\text{dCTP}$ was the rate-limiting substrate, and the other three natural triphosphate substrates were in excess. The Lineweaver-Burk plots were drawn to determine the mode of inhibition. The plots obtained were typical of competitive inhibition with respect to dCTP (Fig. 3); similarly, FMACTP and FMAUTP competed with dCTP and dTTP, respectively. The K_i values were determined by replotting the slopes as shown in the inset of Fig. 3; the K_i values of FMAUTP, FMACTP, and FIACPTP were 0.06, 0.47, and 0.30 μ M, respectively (Table 1). Similar experiments with cellular α polymerase showed

FIG. 1. Further purification of cellular DNA polymerase α and HCMV-induced DNA polymerase on heparin agarose and single-stranded DNA agarose chromatographies. Activated calf thymus DNA was used as the template primer throughout this study. The enzyme fractions of α polymerase and virus-induced DNA polymerase from a phosphocellulose column (15) were pooled separately, dialyzed against buffer A (10 mM KPO_4 [pH 8.0], 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol), and applied to heparin agarose columns. The elution was carried out with a linear gradient of 0.1 to 0.7 M KCl in buffer A. Fractions of 2.0 ml were collected, and 20 μl -volumes from each fraction were assayed for DNA polymerase activity. The α polymerase eluted at about 0.21 M KCl (A), and virus-induced DNA polymerase eluted at 0.32 M KCl (B). The corresponding α and virus-induced enzyme fractions from heparin agarose chromatography were pooled, exhaustively dialyzed against buffer A, and loaded onto the single-stranded DNA agarose column. The elution and enzyme assay conditions were identical to those given above. The α polymerase eluted at 0.11 M KCl (C), and virus-induced enzyme eluted at 0.21 M KCl (D). Virus-induced enzyme activity was enhanced by the presence of 60 mM $(\text{NH}_4)_2\text{SO}_4$ in the reaction mixture ($\circ$ in panel D), and α enzyme was suppressed by the presence of $(\text{NH}_4)_2\text{SO}_4$ ($\circ$ in panel C).

that the mode of inhibition for α polymerase is also of competitive inhibition. DNA polymerase α was much less sensitive to these inhibitors, and its K_i values for FMAUTP, FMACTP, and FIACPT were 0.45, 2.90, and 3.10 μM , respectively (Table 1). Similarly, the K_m values that measured the strength of the affinity of enzymes for their substrates were computed from the data with the absence of inhibitors in assay mixture by drawing Lineweaver-Burk diagrams (Fig. 3). Table 1 shows that HCMV-induced DNA polymerase had an approximately threefold greater affinity for dTTP (K_m , 0.50 μM) and dCTP (K_m , 0.55 μM) than did cellular α polymerase (K_m for dTTP, 1.60 μM ; K_m for dCTP, 1.50 μM).

FMAUTP, FMACTP, and FIACPT as alternate substrates. The DNA polymerases require all four natural deoxyribonucleoside triphosphates for optimal activity. In this study, deletion of dTTP or dCTP from the HCMV DNA polymerase reaction mixture decreased DNA synthesis to approximately 25% of optimal activity (Fig. 4). For α polymerase, omission of dTTP or dCTP from the assay mixture reduced DNA synthesis to about 40% of optimal activity under the specified conditions (Fig. 4). Addition of FMAUTP, FMACTP, or FIACPT as a substituting for its natural

triphosphate counterpart to the reaction mixture enhanced in vitro DNA synthesis catalyzed by HCMV-induced DNA polymerase; a gradual increase in DNA synthesis was in response to the increase of the nucleoside analog triphosphate concentration in the reaction mixture (Fig. 4). In contrast, DNA synthesis catalyzed by α polymerase decreased gradually with the increase of the analog triphosphate concentration in the assay mixture (Fig. 4). These results indicate that the three nucleoside analog triphosphates (FMAUTP, FMACTP, and FIACPT) can be alternate substrates in in vitro DNA synthesis catalyzed only by HCMV-induced DNA polymerase but not by cellular DNA polymerase α .

DISCUSSION

Infection of permissive cells with herpesviruses results in the induction of several virus-specific enzymes. These virally encoded enzymes function to catalyze reactions similar to those mediated by their host cellular counterparts. However, the virus-induced enzymes differ from cellular enzymes not only in their physical and immunological properties but also in their kinetic behavior. Among the herpesvirus-induced enzymes, TK and DNA polymerase were

FIG. 2. The purified HCMV-induced DNA polymerase was analyzed in a 9% sodium dodecyl sulfate-polyacrylamide gel and visualized by silver staining (26). M, Protein markers; V, virus-induced DNA polymerase; k, molecular weights in thousands.

demonstrated to have substrate specificities distinctly different from those of cellular enzymes. Thus these two enzymes have been widely exploited as targets for the design of selective antiherpesvirus drugs (3, 4). Studies on the activities of a series of antiherpesvirus nucleoside analogs against HSV type 1 mutants, at both the TK and DNA polymerase loci, indicate that the virus-induced TK is required for the action of the nucleoside analogs against HSV type 1 replication (7, 10, 11). These nucleoside analogs exerted selective antiherpesvirus activity because of their preferential phosphorylation by the viral TK and because of a preferential interaction of their triphosphates formed in infected cells with the viral DNA polymerase (7, 10, 11). Although HCMV is a member of the herpesvirus group, no evidence has been found that an HCMV-specific TK is present in infected cells (12, 27). Productive replication of HCMV in human fibroblast cells, however, can induce the expression of a virus-specific DNA polymerase (15).

Recently, in two studies (8, 20) it has been demonstrated that FMAU, FMAC, and FIAC are potent inhibitors of HCMV replication, as compared with the existing antiherpesvirus drugs, in spite of the lack of an HCMV-specific TK expression. Our previous study on the antiviral effect, using a sensitive quantitative cRNA-DNA hybridization technique, revealed that these three compounds specifically inhibit viral DNA replication (20). This observation has prompted a speculation that although FMAU, FMAC, and FIAC are restrictively phosphorylated in HCMV-infected cells (8), enough of these drugs may be phosphorylated to the triphosphates by cellular kinase(s) to selectively inhibit the virus-specific DNA polymerase. To explore this

FIG. 3. Competitive inhibition of HCMV-induced DNA polymerase by FIACTP. The reaction mixture (total volume, 100 μ l) contained 20 mM Tris hydrochloride (pH 8.0); 10 mM $MgCl_2$; 0.4 mM dithiothreitol; 20 μ g of BSA; 60 mM $(NH_4)_2SO_4$; 20 μ g of activated calf thymus DNA; 10 μ M each of dATP, dGTP, and dTTP; various concentrations of $[^3H]$ dCTP (30 Ci/mmol); 0.5 U of enzyme; and FIACTP at 0 μ M ($\circ$), 1 μ M (Δ), and 3 μ M ($\square$). The reaction mixture was incubated at 37°C for 30 min. The K_m value for FIACTP was determined by replotting the slopes (inset).

possibility, it required purification of virus-induced DNA polymerase as well as cellular α polymerase for further study. Details of purification and characterization of HCMV-induced DNA polymerase and α polymerase are described in the legend to Fig. 1. It is noteworthy that HCMV-induced DNA differed, in general, from the cellular α enzyme in its affinity for dCTP and dTTP (Table 1). The purified HCMV-induced DNA polymerase consisted of two polypeptide bands corresponding to molecular weights of 140,000 and 58,000 in a denaturing acrylamide gel (Fig. 2). The HCMV DNA polymerase protein pattern was somewhat similar to that of purified HSV DNA polymerase in which two polypeptides with molecular weights of 150,000 and 54,000 were copurified reproducibly (10, 16, 21, 23). In the HSV type 2 system, the polypeptide with a molecular weight of 150,000 is associated with the polymerase activity and the

TABLE 1. Kinetic constants for HCMV-induced DNA polymerase and cellular DNA polymerase

DNA polymerase	K_m (μ M) of ^a :		K_i (μ M) of ^b :		
	dCTP	dTTP	FMAUTP	FMACTP	FIACTP
HCMV	0.55	0.50	0.06	0.47	0.30
Human α	1.50	1.60	0.45	2.90	3.10

^a K_m values were determined by drawing Lineweaver-Burk plots for the indicated triphosphates. Reaction conditions for HCMV-induced DNA polymerase and cellular DNA polymerase α are given in detail in the text.

^b Reactions were carried out as described in the text with FMAUTP, FMACTP, or FIACTP as the inhibitor. For DNA polymerase α , reaction mixtures did not contain $(NH_4)_2SO_4$.

FIG. 4. Examination of FMAUTP, FMACTP, and FIACTP as alternate substrates in DNA syntheses in vitro by HCMV-induced DNA polymerase (A) and cellular DNA polymerase α (B). The assay conditions were similar to those described for the determination of K_m values except that dTTP was substituted by FMAUTP and dCTP was substituted by FMACTP or FIACTP. [³H]dGTP was used throughout these experiments as the labeled substrate: 10 μ M for HCMV DNA polymerase and 100 μ M for α polymerase. For the HCMV DNA polymerase reaction mixture, 100% activity with all four natural triphosphates represents 5.6 pmol of [³H]dGMP incorporation into activated calf thymus DNA. For α polymerase, 100% activity with all four natural triphosphates represents 12.6 pmol of [³H]dGMP incorporation into activated calf thymus DNA.

protein with a molecular weight of 54,000 is an independent gene product from the major component of the DNA polymerase (25). The molecular basis for the HCMV-induced DNA polymerase, however, has not been defined; determination of the genomic location and biochemical characteristics of the two polypeptides identified in this study will require further investigation.

To determine whether FMAU, FMAC, and FIAC are selective at the DNA polymerase level, we tested the interaction of these nucleoside analogs and their triphosphated derivatives FMAUTP, FMACTP, and FIACTP with the HCMV-induced DNA polymerase as well as DNA polymerase α . The results show that low concentrations of these triphosphated derivatives inhibit the virus-induced DNA polymerase (Table 1). However, the unphosphorylated nucleoside analogs have no effect on the virus-induced DNA polymerase even at large concentrations (data not shown). These data imply that FMAU, FMAC, and FIAC must be converted to their triphosphated derivatives before the ac-

tivity of the virus-induced DNA polymerase will be inhibited. We also examined the virus-induced DNA polymerase and α polymerase for their specificities toward utilization of FMAUTP, FMACTP, and FIACTP as alternate substrates in the DNA polymerase reaction. Basically, the DNA polymerases require all four natural deoxyribonucleoside triphosphates for their optimal activity; omission of one of the normal triphosphates from the reaction mixtures results in a remarkable decrease of DNA synthesis. For example, in this study, limited DNA synthesis occurred when dTTP or dCTP was omitted from the DNA polymerase assay mixture (Fig. 4). Once FMAUTP instead of dTTP was added to the virus-induced DNA polymerase assay mixture, DNA increased in response to the increase of the FMAUTP concentration in the reaction mixture. Similarly, the addition of FMACTP (or FIACTP) instead of dCTP to the reaction mixture also enhanced DNA synthesis (Fig. 4). In a similar experiment with DNA polymerase α , substitution of a given analog triphosphate for its respective natural substrate did not enhance DNA synthesis (Fig. 4). These results indicate that FMAUTP, FMACTP, and FIATP are alternate substrates in DNA synthesis catalyzed efficiently by HCMV-induced DNA polymerase but not by DNA polymerase α . These findings are similar to those reported for the HSV type 1 and HSV type 2 systems in which virus-specific DNA polymerases could utilize the triphosphated analogs efficiently as substrates but not the cellular DNA polymerases (1, 22). The data from this study and those from a previous report (20) reveal that the inhibitory effects of FMAUTP, FMACTP, and FIACTP on HCMV-induced DNA polymerase correlate with the antiviral activity of the corresponding nucleoside FMAU, FMAC, and FIAC at the viral DNA replication level in infected cell cultures. These data suggest that the anti-HCMV selectivity of FMAU, FMAC, and FIAC might virtually result from the triphosphated analogs in HCMV-infected cells as mentioned above, although they are formed in low amounts presumably by cellular kinase(s). Alternately, these anti-HCMV drugs might act by a mechanism which does not require phosphorylation in virus-infected cells, i.e., the alternation of the normal deoxyribosynucleoside pool or the inhibition of phosphorylation of the natural deoxyribosynucleosides. Research in this field is under way in our laboratory.

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