Analysis of Deoxycytidine (dC) Deaminase Activity in Herpes Simplex Virus-infected or HSV TK-transformed Cells: Association with Mycoplasma Contamination but Not with Virus Infection

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SUMMARY

Deoxycytidine (dC) deaminase activity has been previously reported to be induced in herpes simplex virus (HSV)-infected cells (Chan, 1977). In contrast, we report here that HSV infection of either hamster cells naturally deficient in this enzyme activity or mouse cells containing a low level of activity never resulted in appearance or stimulation of dC deaminase, whereas thymidine kinase (TK) was always induced. Surprisingly, dC deaminase activity, which differed by electrophoretic mobility from the mouse or human cell enzyme, was discovered in some cells selected for the presence of HSV TK after infection with u.v.-irradiated HSV. Evidence is presented which suggests that the appearance of this new enzyme was not due to the presence of virus genes but rather to mycoplasma contamination.

Several new enzyme activities involved in DNA metabolism appear in herpes simplex virus (HSV)-infected cells soon after infection. Among them, DNA polymerase (Weissbach et al., 1973; Jofre et al., 1977; Purifoy et al., 1977), DNase (Hoffman & Chang, 1978; Moss et al., 1979) and thymidine kinase (TK) (Summers et al., 1975; Cheng & Ostander, 1976; Kit et al., 1976) have been demonstrated to be coded by the virus genome. The TK gene can be transferred to TK− cells (Munyon et al., 1971) thus permitting these cells to survive in selective medium containing hypoxanthine, aminopterin and thymidine (HAT medium) (Littlefield, 1964). Other enzymes have also been reported to be induced upon infection by HSV, namely, a deoxycytidine (dC) deaminase (Chan, 1977) and a ribonucleotide reductase (Cohen, 1972; Langelier et al., 1978) which, if they were virus-coded, could be used as additional selective markers.

As numerous established cell lines are naturally deficient in dC deaminase activity (Camiener & Smith, 1965; Tomchick et al., 1968; Cooper, 1973; Mayers et al., 1973) and a selection method has been devised to select dC deaminase-positive cells (Chan, 1977; Langelier et al., 1978), it was very attractive to test the possibility of transfer of the HSV-induced dC deaminase into some of these deficient cells. However, as we had already found (Langelier et al., 1978) for the previously reported dCMP deaminase induction (Rolton & Keir, 1974), we have been unable to confirm the induction of dC deaminase in HSV-infected cells. On the other hand, we discovered that in some of our HSV TK-transformed cell lines the appearance of dC deaminase activity could be associated with the presence of mycoplasma and we have performed experiments which suggest that mycoplasma contamination of virus stocks could explain the reported HSV induction of dC deaminase.

The cell lines used in this study were cultivated in a modified Eagle’s minimum essential medium supplemented with 10% foetal calf serum as previously described (Langelier et al., 1978). HSV-1 strains F, A-44, B-12, G-24, KOS and HSV-2 strain MS were obtained from P. Sheldrick (Villejuif) and HSV-1 strain 17MP and HSV-2 strain HG-52 were obtained from J.
Subak-Sharpe (Glasgow). Virus stocks were prepared and titrated as in our previous report (Langelier et al., 1978).

For the dC deaminase assay, confluent cultures of infected or uninfected cells washed twice with phosphate-buffered saline were suspended in a small volume of 50 mm-tris-HCl pH 7.8, 1 mm-dithiothreitol, 1 mm-EDTA and extracted by sonication. The incubation mixture contained in a final vol. of 40 μl 0.1 m-tris-HCl pH 7.8, 0.2 mm-dC plus [5-3H]dC (1.2 × 10⁶ ct/min) and enzyme extract (20 to 400 μg protein). The mixture was incubated at 37 °C for 30 min and the reaction was stopped by immersing the tubes in boiling water for 2 min. After centrifugation at 10,000 g for 10 min to remove the precipitate, the product (dU) was separated from the substrate (dC) by unidimensional migration on polyethyleneimine-cellulose sheet in ethyl acetate : isopropanol : water (117:41.1:21.9). In some experiments, dC deaminase activity was measured with EDTA in the incubation mixture and with separation of dU from dC by column chromatography exactly as reported by Chan (1977). The enzyme activities obtained by the two methods agreed well. The TK and ribonucleotide reductase assays were performed as previously reported (Langelier et al., 1978).

To separate cellular dC deaminase activity from the mycoplasma isozyme, polyacrylamide gel electrophoresis (PAGE) was performed on cellular extracts centrifuged at 100,000 g for 1 h essentially as described by Kit et al. (1973) except that dT, MgCl₂ and mercaptoethanol were omitted from the gels and the buffers. Electrophoresis was run at 4 °C, at 4 mA/gel. After 5 cm migration of the tracking dye, the gels were cut with a gel slicer into 2-mm sections that were incubated overnight at 37 °C with 150 μl of assay mixture.

Since Chinese hamster (CCL39 and its derivatives) or Syrian hamster (BHK-21/C13) cells are naturally deficient in dC deaminase activity (Cooper, 1973; Robert de Saint-Vincent et al., 1980), and since the HSV-induced enzymes (TK, ribonucleotide reductase and DNA polymerase) were easily detectable in these cells (Langelier et al., 1978; S. Qualizza & Y. Langelier, unpublished results), they were used as host cell lines to look for dC deaminase activity after HSV infection. In numerous experiments, using different strains of HSV-1 (A-44, 17MP, B-12, KOS, F and G-24) and of HSV-2 (HG-52 and MS) for the infection of GMA32 [a cell line derived from CCL39 lacking dC kinase (dCK-)] or BHK-21/C13 cell line, dC deaminase activity was never observed above the limit of sensitivity of our assay (0.005 nmol/min/mg). TK and ribonucleotide reductase activities were often measured to verify the expression of the HSV genome and they were always induced. Variations in the time of infection (from 2 to 36 h) or in the m.o.i. (0.1 to 100) did not produce any appearance of dC deaminase activity. Moreover, similar experiments were performed using a mouse cell line LM (TK-) clone 1D (Kit & Dubbs, 1963) which expresses a low level of cellular dC deaminase activity (0.35 to 0.40 nmol/min/mg). Infection by HSV did not increase the activity. Our results and similar observations done in two other laboratories (S. Bachetti, personal communication; Y.-C. Cheng, personal communication) raised the question as to whether dC deaminase could indeed be significantly affected by HSV infection.

During experiments on HSV TK-transformed hamster cell lines obtained after infection of GMAFa1 (TK-, dCK-) cells by u.v.-irradiated HSV, we carefully checked for dC deaminase activity which, if present, could modify the results of our studies on [5-3H]dC incorporation into DNA (J. Charron & Y. Langelier, unpublished results). Surprisingly, we found that one transformed cell line Fa1-HG-20 had a high level of dC deaminase activity, whereas the parental and another HSV TK-transformed line Fa1-HG-21 did not (Table 1). The dC deaminase activity was also absent in a series of transformed cell lines derived from GMF11, another TK- cell line. On the other hand, an increase in dC deaminase activity was observed in some transformed cell lines (e.g. 1D-52-15b) derived from LM (TK-) clone 1D cells, which express a low level of enzyme activity.

Before pursuing experiments to explain these results, we tested all our cell lines for the
Table 1. dC deaminase activity in different cell lines uncontaminated or contaminated by mycoplasma

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>dC deaminase activity (nmol/min/mg)</th>
<th>Cytoplasmic Hoechst fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMAF1*</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td>Fa1-HG-20†</td>
<td>1.25</td>
<td>+</td>
</tr>
<tr>
<td>Fa1-HG-21†</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td>GMF11*</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td>F11-52-C1, C3, C5, C13‡</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td>LM clone 1D</td>
<td>0.35</td>
<td>-</td>
</tr>
<tr>
<td>1D-52-15b§</td>
<td>1.83</td>
<td>+</td>
</tr>
<tr>
<td>HeLa</td>
<td>8.6</td>
<td>+</td>
</tr>
<tr>
<td>BHK-21/C13</td>
<td>&lt;0.005</td>
<td>-</td>
</tr>
<tr>
<td>BHK-21/C13</td>
<td>0.3</td>
<td>+</td>
</tr>
</tbody>
</table>

* GMAF1 and GMF11 are respectively TK−, dCK− and TK− mutant cell lines selected from CCL39 as described previously (Robert de Saint-Vincent et al., 1980).
† Two cell lines selected in HAT medium after transformation of GMAF1 by u.v.-irradiated HSV-2 (strain HG-52) using the conditions described by Munyon et al. (1971).
‡ Four lines derived from GMF11 by transformation as indicated in footnote †.
§ One transformed line derived from LM (TK−) clone 1D as indicated in footnote †.

presence of mycoplasma using the sensitive Hoechst staining technique described by Chen (1977). The validity of the Hoechst staining test has been ascertained in some experiments by electron microscopy and by autoradiography of fixed cells after growth in medium containing [5-3H]thymidine. As shown in Table 1, four cell lines were contaminated by mycoplasma, among which were the two transformed lines with an increase in dC deaminase activity. HeLa cells, as previously described for other human cell lines (Camiener & Smith, 1965; Chan, 1977), expressed a high level of dC deaminase activity and were also contaminated by mycoplasma. The results with BHK-21/C13 cells obtained from different sources also indicated that contamination by mycoplasma could result in the appearance of the enzyme. Furthermore, when contaminated BHK-21/C13 and Fa1-HG-20 cells were grown for 4 days in the presence of 60 μg/ml tylocine (Gibco), an anti-mycoplasma agent, the dC deaminase activity completely disappeared. This treatment did not affect the level of cellular dC deaminase activity present in LM (TK−) clone 1D cells (data not shown).

Therefore, as has been previously shown for other enzymes such as hypoxanthine phosphoribosyltransferase (Van Diggelen et al., 1977), thymidine or uridine phosphorylases (Van Roy & Fiers, 1977) and adenosine phosphorylase (Hatanaka et al., 1975), we postulated that mycoplasma contamination could introduce a new dC deaminase activity in cultured cells. More direct evidence for this hypothesis was obtained using PAGE to separate the mycoplasma dC deaminase from the cellular one. The results of electrophoresis performed on extracts of cell lines expressing dC deaminase activity are shown in Fig. 1 and indicate that two migrating forms of the enzyme existed only in mycoplasma-contaminated cells expressing cellular activity (HeLa and 1D-52-15b cells). By comparison with electrophoretic profiles of extracts of contaminated hamster Fa1-HG-20 cells devoid of cellular activity, it was concluded that the hypothetical mycoplasma enzyme migrated more slowly ($R_M$ 0.5 to 0.52) than the cellular one ($R_M$ 0.8 for HeLa and 0.96 for 1D-52-15b). In extracts of mycoplasma-free LM (TK−) clone 1D cells, the peak with an $R_M$ of 0.5 was absent. On the other hand, this peak was present in extracts of pelleted material obtained by centrifugation (30,000 g for 40 min) of Fa1-HG-20 cell culture medium filtered through a 0.22 μm membrane.

In order to establish that mycoplasma could, by contamination, introduce its dC deaminase activity into dC deaminase-deficient cells, experiments of deliberate contamination were
performed. BHK-21/C13 cells were contaminated by (i) culture medium, with or without filtration through a 0.22 μm membrane, in which Fa1-HG-20 cells were grown to confluence, or by (ii) cellular extracts of the same cell cultures given the same treatment used to prepare virus stocks (3 cycles of freezing and thawing). Mock-contamination was done in parallel using culture medium or cellular extracts of uncontaminated cells. The cell cultures were grown to confluence and the dC deaminase activity was measured and analysed by PAGE. The results indicated unequivocally that dC deaminase activity with an $R_M$ of 0.5 could be transferred to dC deaminase-deficient BHK-21/C13 cells by both the culture medium (either filtered or not) and by the cellular extract of contaminated Fa1-HG-20 cells, and that mycoplasma became simultaneously associated with the cells as detected by cytoplasmic Hoechst staining. Identical results were obtained after two passages of the contaminated cultures. Mock-contaminated cultures were negative for the presence of dC deaminase and mycoplasma. Finally, BHK-21/C13 cells were deliberately contaminated with a reference strain of mycoplasma, Mycoplasma hyorhinis. When examined after one passage, the contaminated cultures were found to be positive for cytoplasmic Hoechst staining and for dC deaminase with an $R_M$ of 0.53.

The results presented in this study indicate that infection by HSV did not produce the appearance of a new dC deaminase activity in hamster cells devoid of this activity, nor did it stimulate the cellular enzyme in mouse cells. Moreover, our observation that mycoplasma-associated dC deaminase activity could be deliberately transferred to dC deaminase-negative cells using conditions mimicking infection by mycoplasma-contaminated virus stocks suggests that mycoplasma present in the virus stocks used for infection in Chan's experiments (1977) could have produced the induction of dC deaminase. From the data presented in this report, this hypothesis cannot be ruled out; (i) the Vero cells used to propagate Chan's virus may not have been tested for mycoplasma contamination; (ii) Chan's BHK-21/C13 cells exhibit a dC deaminase activity without HSV infection and thus appear to be contaminated by mycoplasma.

Some other indirect evidence that dC deaminase is not induced in HSV-infected cells has been provided by the work of Aswell & Gentry (1977). They have found that 5-methyl-ara C inhibited HSV production only in cells containing dC deaminase activity (e.g. human cells). In such cells, 5-methyl-ara C can be deaminated to ara-T, which can then be phosphorylated by the viral TK to a form inhibitory to DNA replication (Aswell & Gentry, 1976). The synthesis of a virus-coded dC deaminase would be expected to produce an HSV sensitivity to 5-methyl-ara C independent of the presence of the cellular isozyme.
Finally, our finding that mycoplasma contamination could introduce enzyme activities which modify the cellular phenotype strengthens the importance of maintaining cell cultures and virus stocks free of mycoplasma, especially in studies on virus-induced enzymes.

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