Effect of Actinomycin D and Cycloheximide on Epstein-Barr Virus Early Antigen Induction in Lymphoblastoid Cells

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SUMMARY

The effect of inhibitors of RNA (actinomycin D, Act. D) and protein synthesis (cycloheximide, CH) on induction of Epstein-Barr virus (EBV) antigens by the tumour promoter TPA and by EBV superinfection has been analysed. The data indicate that (i) concomitant treatment of cells with Act. D and TPA leads to complete suppression of virus antigen induction. Subsequent treatment of the cells with Act. D after prior exposure to TPA results in some virus antigen induction, the amount depending on the time of TPA treatment. (ii) Simultaneous treatment of the cells with TPA and CH blocks antigen expression completely. Removal of the inhibitor results in antigen expression at a comparable rate to that of CH-untreated cells. (iii) If CH treatment is followed by addition of Act. D, virus antigen induction by TPA is completely inhibited. In contrast, superinfection of the cells with P3HR-1 EBV in the presence of CH for the same period followed by removal of the inhibitor and addition of Act. D leads to virus antigen expression by 3 h after Act. D addition. (iv) Concomitant treatment with CH and TPA followed by addition of either iododeoxyuridine or n-butyric acid results in 'superinduction'. Virtually all cells exhibit EBV-specified antigens. This implies that induction of virus antigens by tumour promoters requires the synthesis of a specific RNA, that this RNA increases in concentration during the induction period and that the same RNA is not required for EBV transcription after exogenous infection.

INTRODUCTION

Addition of a variety of reagents such as halogenated thymidine analogues, tumour promoters, antisera to human IgM and n-butyric acid to cells of Epstein-Barr virus (EBV) non-producer lines causes induction of EBV-specific early antigen (EA) synthesis (Gerber, 1972; Hampar et al. 1972; Sugawara et al. 1972; zur Hausen et al. 1978; Tovey et al. 1978; Luka et al. 1979). In such cells, appearance of virus structural proteins is only observed after superinfection with P3HR-1 EBV (Gergely et al. 1971; Klein et al. 1972; Yajima & Nonoyama, 1976; Fresen et al. 1978) and it has been proposed that the exclusive induction of virus-specific early proteins in these non-producer lines results from the presence of defective endogenous EBV genomes (Fresen et al. 1978). The mechanism of expression and suppression of EBV genomes in lymphoblastoid cells is poorly understood at present.

Previously we have shown that (i) the efficiency of chemical induction but not of superinfection correlates with the number of virus genome copies/cell in non-producer Raji cells (Bister et al. 1979). (ii) Induction by TPA but also by iododeoxyuridine (IdUrd) and by anti-IgM sera is largely inhibited by retinoic acid, without affecting EBV genome expression after superinfection (Yamamoto et al. 1979). (iii) Treatment of Raji cells with TPA did not
change the ratio of EBV-specific DNA to cellular DNA (Bister et al. 1979). This implies that a common mechanism acts in the induction by chemical inducers which is independent of mechanisms regulating exogenous infection. In addition, induction does not necessarily involve replication of virus DNA.

More recently, we have studied the effects of several inhibitors of DNA synthesis on induction by tumour promoters (Yamamoto & zur Hausen, 1980). Cytosine arabinoside completely inhibits cellular DNA synthesis without affecting the expression of early virus functions under conditions of concomitant TPA treatment.

The present experiments were undertaken to study the effect of inhibition of RNA and protein synthesis on EA induction by chemical reagents as well as by exogenous virus infection.

**METHODS**

*Cells and viruses.* The origin and maintenance of EBV genome-negative BJA-B cells, of EBV genome-positive non-producer NC37 cells and the virus-producer P3HR-1 line have been described previously (Fresen & zur Hausen, 1976). These cell lines were cultivated in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% foetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Conditions for the induction of EBV by TPA have been described (Bister et al. 1979). Briefly, 10 to 15 ml of cell suspensions (about 5 x 10⁵ cells/ml) were treated with TPA dissolved in dimethyl sulphoxide. Unless otherwise stated, 20 ng/ml TPA were used throughout the experiments. Conditions for the differential treatment of the cells with Act. D and/or CH and for superinduction are described in detail in the legends to the figures. Infectious EBV was obtained from P3HR-1 cells by differential centrifugation and concentrated about 100-fold from supernatants of aged cultures.

*Chemicals.* TPA, cytosine arabinoside (Ara-C), fluorodeoxyuridine (FdUrd), 5′-iodo-2′-deoxyuridine (IdUrd), n-butyric acid, CH and Act. D were purchased from Sigma, St. Louis, Mo., U.S.A. Concentrations used in the experiments are indicated in the text.

*Demonstration of EBV-specific EA.* EA induction was monitored by indirect immunofluorescence as described previously (Henle & Henle, 1966; zur Hausen et al. 1979; Yamamoto et al. 1979).

**RESULTS**

*Inhibition of RNA synthesis during the induction period*

These experiments were designed to investigate whether TPA stimulates EA production in the absence of RNA synthesis. Act. D was used to inhibit RNA transcription and was added (0.25 μg/ml) to NC37 cells simultaneously with TPA or 1, 2, 3 or 4 days after its addition (Fig. 1). Samples were collected after different times of incubation and examined for the percentage of EA-producing cells by indirect immunofluorescence. The results indicate that concomitant addition of inhibitor and TPA resulted in complete inhibition of EA induction. However, if the inhibitor was added at intervals after prior induction, some EA induction occurred which correlated with the inhibitor-free time period. Act. D added 3 days after induction had no effect on subsequent EA expression. This suggests that sufficient EA-specific message has been accumulated by 2 days but that EA expression is not influenced by Act. D addition after this period.

*Inhibition of protein synthesis during induction*

The next series of experiments was designed to study whether EA induction by TPA as well as by superinfection depends on prior synthesis of non-EA proteins. Cells were incubated with TPA and CH (50 μg/ml) for various periods of time (2, 3 and 5 days), washed
EBV early antigen induction by tumour promoters

Fig. 1. Effect of Act. D added at various times after TPA induction: NC37 cell suspensions (10 ml at $5 \times 10^5$/ml) were incubated with 20 ng/ml TPA at zero time. Act. D (0.25 μg/ml) was added at various intervals: ○-○, no addition; △-△, addition at day 0; ■-■, addition after 1 day; □-□, addition after 2 days; ●-●, addition after 3 days; Δ-Δ, addition after 4 days. Samples were removed and assayed for EA induction as described by Yamamoto & zur Hausen (1979).

Fig. 2. Effect of different exposure times of NC37 cells to CH on appearance of EA. Cells were treated with TPA and 50 μg/ml CH at zero time. After the indicated times of exposure (●-●, 2 days; ■-■, 3 days; Δ-Δ, 5 days) to CH and TPA, cells were washed three times with PBS and resuspended in complete medium without CH and TPA. The percentage of EA-positive cells was determined during a 7 day period following removal of the inhibitor.

Fig. 3. Effect of CH and Act. D on EA induction by TPA. NC37 cells were treated with TPA and CH at zero time (50 μg/ml). After 2 days they were washed three times with PBS, then further incubated with or without Act. D (0.25 μg/ml): ○-○, medium only; □-□, Act. D; ●-●, TPA; ■-■, Act. D + TPA. At the times shown EA synthesis was monitored by indirect immunofluorescence.

Thoroughly three times with phosphate-buffered saline (PBS) and then further incubated. Previous studies revealed that EA induction by TPA resulted in the appearance of the first antigen-positive cells about 1 day after addition of the promoter (also shown in Fig. 1). In such cultures the highest percentage of induced cells was obtained about 5 days after induction. Fig. 2 clearly shows that the kinetics of EA induction after CH treatment and removal of the inhibitor followed a very similar pattern to that of non-CH-treated cultures, irrespective of the initial incubation period with CH.
Fig. 4 Effect of CH and Act. D on EA induction by superinfection or infection with P3HR-1 EBV. BJA-B and NC37 cells were infected with P3HR-1. Adsorption was carried out at 37 °C for 1 h. The cells were then treated with 50 µg/ml CH at zero time. After incubation for 2 additional days, cells were washed three times with PBS and incubated with Act. D (50 µg/ml) (○—○, BJA-B cells; ■—■, NC37 cells) or without inhibitor (○—○, BJA-B cells; □—□, NC37 cells). Samples were collected daily and examined for EA synthesis.

Fig. 5 Superinduction of EA by IdUrd and butyric acid. NC37 cells were pretreated with TPA and 0.67 µg/ml CH at zero time for 3 days. Cells were then washed three times with PBS and incubated with or without inducing drugs for 3 additional days: ○—○, medium alone; △—△, 50 µg/ml IdUrd, □—□, 3 mM butyric acid; ●—●, TPA.

In a second set of experiments NC37 cells were treated concomitantly with TPA and CH (50 µg/ml) for 2 days, washed three times with PBS and then divided into four portions. Subsequently they were treated with or without Act. D (0.25 µg/ml) and/or TPA respectively. As shown in Fig. 3, the culture which received inhibitor-free medium showed a gradual increase in EA-positive cells. The addition of Act. D, however, abolished the appearance of EA-positive cells completely, irrespective of the presence or absence of TPA. Interestingly, addition of fresh TPA did not cause any further increase in the percentage of EA-positive cells.

Additional experiments were carried out without using TPA but by superinfecting NC37 and BJA-B cells with P3HR-1 EBV (Fig. 4). After 2 days, non-adsorbed virus and inhibitor were removed by washing followed by Act. D treatment as described above. In these tests a prompt increase in EA-positive cells occurred after removal of CH independent of the presence or absence of Act. D. Only 3 h after CH removal definite induction of EA was visible, reaching a maximum 1 day after addition of Act. D. Both types of cells provided the same data although there was a significant difference in the percentage of induced cells, as observed in earlier experiments (zur Hausen & Fresen, 1977).

Superinduction of EA

We investigated the possibility of superinduction of EA by TPA and by various inhibitors of macromolecular synthesis. NC37 cells were treated with TPA for 2 days, then several inhibitors of macromolecular synthesis were added at concentrations indicated in Table 1, without washing the cells. After 2 additional days cells were analysed for EA synthesis. The
Table 1. Superinduction of EA by various inhibitors of macromolecular synthesis*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Superinduction</th>
</tr>
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<tbody>
<tr>
<td>Act. D</td>
<td>10, 5, 1 (μg/ml)</td>
<td>No</td>
</tr>
<tr>
<td>CH</td>
<td>50, 5, 0.5 (μg/ml)</td>
<td>No</td>
</tr>
<tr>
<td>Ara-C</td>
<td>20, 2, 0.2 (μg/ml)</td>
<td>No</td>
</tr>
<tr>
<td>IdUrd</td>
<td>100, 10, 1 (μg/ml)</td>
<td>Yes (40.1, 50.9, 30.0)†</td>
</tr>
<tr>
<td>n-butyric acid</td>
<td>30, 3, 0.3 (mM)</td>
<td>Yes (11.5, 36.3, 26.4)†</td>
</tr>
<tr>
<td>FdUrd</td>
<td>20, 2, 0.2 (μg/ml)</td>
<td>No</td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>50, 5, 0.5 (μg/ml)</td>
<td>No</td>
</tr>
<tr>
<td>TPA only</td>
<td>20 ng/ml</td>
<td>13.0†</td>
</tr>
<tr>
<td>IdUrd only</td>
<td>100, 50, 10 (μg/ml)</td>
<td>10-14†</td>
</tr>
<tr>
<td>n-butyric acid only</td>
<td>3 (mM)</td>
<td>8-6†</td>
</tr>
</tbody>
</table>

* NC37 cells were treated with TPA for 2 days, then inhibitors were added at the indicated concentrations. Three days after addition of the inhibitors, samples were collected for EA synthesis.† % of EA-positive cells.

results indicated that the three concentrations of IdUrd used and two lower concentrations of butyric acid further enhanced EA induction by TPA. The extremely high induction was not caused by a simple additive effect of these chemicals since without TPA both drugs resulted in about 9 to 13% of EA-positive cells respectively. However, no synergistic effect on EA induction was observed in the cultures treated with other inhibitors in addition to TPA.

In order to obtain more information on superinduction, IdUrd and butyric acid were investigated further. Cells were pretreated with TPA and CH [0.67 μg/ml; the concentration found to be the minimum concentration exhibiting complete inhibition of EA induction by TPA (N. Yamamoto et al., unpublished observation)] for 3 days. After washing the cells, they were divided into four parts to which media containing IdUrd (50 μg/ml), butyric acid (3 mM) or TPA were added respectively. Determination of EA was carried out 1 day and 3 days after washing. Fig. 5 clearly reveals the very efficient induction obtained by the addition of IdUrd and butyric acid. In the case of IdUrd treatment most of the cells showed EA synthesis. In agreement with the experiments shown in Fig. 3, addition of fresh TPA did not enhance further EA induction.

DISCUSSION

Tumour promoters induce DNA replication and particle production of oncogenic herpesviruses (zur Hausen et al. 1978, 1979). The investigation of the mechanism of virus induction may shed some light on the control of persisting herpes virus genomes. The availability of cells carrying the EBV genome, which can be only abortively induced to EA synthesis exclusively (Raji and NC37 cells), offers the opportunity to study the dependence of virus induction on replication of cellular and virus DNA. Inhibition of RNA synthesis by Act. D in the presence of TPA completely prevented EA induction, revealing the requirement of RNA synthesis for EA induction. Subsequent treatment of the cells with Act. D, preceded by TPA addition for various intervals, showed that a period of approx. 3 days is required before Act. D-mediated inhibition of RNA synthesis is without effect on EA induction. This suggests that a specific messenger RNA accumulates during the first 2 days following TPA treatment, then initiating EA synthesis.

Without inhibitors of RNA synthesis, TPA starts to induce EA approx. 24 h after treatment, a maximum effect being reached 5 to 6 days after TPA exposure (zur Hausen et al. 1978; Yamamoto et al. 1979; Yamamoto & zur Hausen, 1980). Therefore, it was of interest to determine whether the initial inhibition of protein synthesis for various periods of time by CH, followed by release of this block, would permit the quick appearance of EA-positive cells. The data show, however, that even 5 days of simultaneous TPA and CH
addition followed by removal of the inhibitor resulted in approximately the same kinetics of EA induction as observed in cells induced without the protein inhibitor. Maximum EA induction occurred about 5 days after removal of the inhibitor. This suggests that synthesis of a specific protein precedes induction of EA synthesis.

This interpretation is supported by experiments inhibiting initially CH-treated TPA-induced cells subsequently with Act. D. Under these conditions EA induction is totally suppressed in Raji and NC37 cells, implying the induction of a non-EA protein before transcription of EA-specific messenger RNA. It is remarkable in this respect that super-infection with P3HR-1 EBV during initial CH treatment, followed later by removal of the drug and by Act. D addition, did not result in complete inhibition of EA synthesis. This strongly suggests that the initial CH treatment did not block transcription of the super-infecting EBV genomes and supports the concept that induction of endogenous EBV genomes is regulated differently to that of superinfected EBV DNA. This is in line with previous data showing the dependence of virus induction on the number of genome copies (Bister et al. 1979), whereas the response to superinfection is independent of the number of endogenous genomes. In addition, retinoic acid (Yamamoto et al. 1979) and L-canavanine (N. Yamamoto, unpublished data) exclusively inhibit virus induction without affecting EA induction by superinfection.

Another approach to studying the control of persisting EBV genomes is represented by 'superinduction' experiments. Tomkins et al. (1972) have summarized a large number and variety of systems in which superinduction by inhibitors of macromolecular synthesis has been reported. In their experiments, e.g. induction and de-induction of tyrosine aminotransferase (TAT), RNA synthesis was required (Garren et al. 1964; Thompson et al. 1970). This led them to propose a post-transcriptional control model for inducible enzymes by postulating the presence of regulator gene product(s). Unlike TAT induction by dexamethasone, EA induction in our system continues for considerable periods even after attempts to remove the inducer by washing. This makes it difficult to decide whether a similar kind of post-transcriptional control acts in EA induction. Superinfection has also been observed in another laboratory (G. Lenoir, personal communication), but was only achieved by additional application of IdUrd and butyric acid which themselves represent efficient inducers of persisting virus DNA. This result suggests that the mechanism of virus induction by TPA is different from that by IdUrd and butyric acid. The kinetics of EBV antigen induction by TPA differ to some extent from IdUrd and butyric acid induction, which induce efficiently within 48 h (Gerber, 1972; Hampar et al. 1972; zur Hausen et al. 1978). When the cells were treated with TPA and CH followed by IdUrd or butyric acid, much higher induction of EA was obtained as compared to the results obtained with simultaneous treatment with TPA and IdUrd or butyric acid (Table 1 and Fig. 5). We do not know whether this more effective induction in CH-treated cells is due to the synchronization of cells triggered in different ways to induce EA after CH treatment. The inhibition of virus induction by all these drugs, as well as by retinoic acid and L-canavanine, shows that even though there must be differences in the inducing mechanism, there must be at least one common step in virus induction.

Our data point to the existence of an efficient and complex controlling system for persisting EBV DNA which does not act on superinfecting EBV DNA entering the same cell.

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REFERENCES


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