Appearance of Early and Late Components of Epstein–Barr Virus-Associated Membrane Antigen in Daudi Cells Superinfected with P3HR-1 Virus

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SUMMARY

This study investigated the synthesis of membrane antigen (MA) as well as virus capsid antigen (VCA) and early antigen (EA) in Daudi cells which had been superinfected with the P3HR-1 strain of Epstein–Barr virus (EBV) and then treated with trypsin to remove initially absorbed MA-positive material from the cell surface. Synthesis of MA, VCA and EA was completely inhibited by puromycin. A marked reduction in the frequency of MA positive cells was observed in superinfected cells cultured in the presence of either cytosine arabinoside (Ara-C) or phosphonoacetate (PA); however, a small fraction of MA synthesis occurred, suggesting an inhibitor insensitive component in MA. A differential absorption of EBV antibody-positive human serum with the Ara-C treated or untreated infected cells detected two antigenically different components in MA: early (Ara-C insensitive) and late (Ara-C sensitive) MA.

INTRODUCTION

Human lymphoblastoid cell lines carrying Epstein–Barr virus (EBV) contain multiple copies of the virus genome (zur Hausen & Schulte-Holthausen, 1970; Nonoyama & Pagano, 1971). Four different types of EBV-determined antigens have been demonstrated by immunofluorescence; these include ‘membrane antigen’ (MA) expressed on the outer surface of viable cells (Klein et al. 1968, 1969), ‘viral capsid antigen’ (VCA) present inside virus-producing cells (Henle & Henle, 1966; zur Hausen et al. 1967), ‘early antigen’ (EA) demonstrated in superinfected Raji, 64–10 cells (Henle et al. 1970) or NC-37 cells (Hinuma, Sairenji & Ohota-Hatano, 1970) and the ‘EBV-determined nuclear antigen’ (EBNA; Reedman & Klein, 1973).

Originally, MA was demonstrated in Burkitt lymphoma biopsy cells (Klein et al. 1966) but can be detected on certain EBV genome-carrying lymphoid cell lines (Klein, Dombos & Gothoskar, 1972). The formation of MA has also been demonstrated on non-producer lines (Raji, 64–10 or NC-37) experimentally superinfected with P3HR-1 strain of EBV (Gergely, Klein & Ernberg, 1971; Pearson, Henle & Henle, 1971; Sairenji & Hinuma, 1975), but not with a transforming EBV strain (Dölken & Klein, 1976). MA appears at an early stage of the virus cycle and is apparently uninfluenced by DNA inhibitors (Gergely et al. 1971; Sairenji & Hinuma, 1975; Dölken & Klein, 1976).
The MA has been divided into at least three subsites distinguished on the basis of antigenic specificity (Svedmyr et al. 1970), as revealed by differential blocking tests with different human sera. More recently Ernberg et al. (1974) subdivided MA in two subclasses, designated early MA (EMA) and late MA (LMA) that differ with respect to their sensitivity to inhibitors of DNA synthesis and their antigenic specificity. LMA is known to be expressed on the surface of VCA positive cells in producer cell lines (Ernberg et al. 1974; Silvestre et al. 1974). Both LMA and EMA have been detected on the envelope of extracellular EB virions (Silvestre et al. 1974). However, it is unknown whether there are any cell lines which permit development of LMA upon superinfection. Detection of LMA following superinfection is important for the evaluation of permissiveness of host cells and the identification of EBV-genome function.

The present investigation provides evidence that the two components of MA, EMA and LMA, are synthesized de novo in Daudi cells superinfected with P3HR-1 virus.

**METHODS**

**Cells.** The Daudi cell line (Nadkarni et al. 1969) originated from a Burkitt lymphoma and was used as target for EBV infection in these studies. The cells contain a small fraction (< 1 %) of VCA-positive cells. They have been maintained in RPMI 1640 medium containing 10 % foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. For the experiments, cells were passaged at 3 to 4 day intervals at a concentration of 2 × 10⁶ cells/ml. Under these conditions at 37 °C the cells showed continued exponential growth and high viabilities (> 98 %) as determined by the trypan blue exclusion method. These passages maintained the fraction of EBV-MA positive cells at a level less than 1 % as tested by membrane immunofluorescence.

**Virus.** Preparations of P3HR-1 strain of EBV (Hinuma et al. 1967) were obtained from media cultured for 14 to 18 days at 33 °C as described (Nagoya & Hinuma, 1972). Unconcentrated or concentrated virus preparations were passed through a 0.8 μm Millipore filter and stored at −80 °C until use.

**Procedure for virus infection and trypsin-treatment.** Daudi cells were mixed with virus to give a final concentration of 2 × 10⁶ cells/ml and incubated at 37 °C for 1 or 2 h. After being washed twice with serum-free Eagle's MEM or Hanks' BSS, the cells were suspended in 0.1 % trypsin (Difco, 1:250) at a concentration of 5 × 10⁶ cells/ml. The mixture was incubated at 37 °C for 30 min with brief shaking every 5 min and then chilled to 0 °C. A 1/10 volume of foetal calf serum was added and the mixture was centrifuged. The cells were resuspended in fresh medium to give a cell concentration of 10⁶ cells/ml and then incubated at 37 °C. The present procedure of trypsin-treatment has been confirmed to greatly reduce the frequency of MA-positive cells due to adsorption of the virus on the cell surface (see Fig. 1) but without interfering with the appearance of EBV-related antigens in the cells (Sairenji & Hinuma, 1975).

**MA assay.** MA was determined by the indirect membrane immunofluorescence method of Klein et al. (1966). The cells were washed twice with phosphate buffered saline, pH 7.2 (PBS) and reacted with a 1:10 or 1:20 dilution of a healthy adult serum, VO-7, unless otherwise stated. The serum had titres of 1:160 in both anti-VCA and anti-MA (Takahashi & Hinuma, 1970), and < 1:5 of anti-EA. This reaction occurred at 37 °C for 30 min and then the cells were washed twice with PBS. The cells were reacted with 1:40 dilution of anti-human IgG (gamma chain specific) FITC-conjugate (Hyland Laboratory, Los Angeles, Calif., U.S.A.) at 37 °C for 30 min as described by Takahashi & Hinuma (1970). After
EBV-membrane antigen

washed with PBS twice, the cells were resuspended in a small volume of 50% glycerin in PBS. One drop of the suspension was taken on a glass slide, covered with a coverslip and then examined with a Chiyoda microscope equipped with a vertical Ploem-type illuminator. Fluorescent cells with more than one dot were judged as MA+ cells. In each sample 300 to 500 cells were counted.

EA and VCA assays. EA+ or VCA+ cells were detected by indirect immunofluorescence using acetone-fixed cell smears as described previously (Ida, Sairenji & Hinuma, 1972). Serum from a patient with nasopharyngeal carcinoma (anti-EA titre, 1:320) was used as EA antibody. VCA was detected by use of the VO-7 serum. The fluorescent antibody used was a goat anti-human IgG specific for H and L chains (Hyland Laboratories).

Absorption of antiserum. The MA-reactive serum, VO-7, was absorbed with the EBV-superinfected Daudi cells (97% MA+) or with EBV-superinfected and Ara-C (20 μg/ml)-treated Daudi cells (76% MA+). Both were harvested 24 h after infection. Cells (5 × 10^7) washed twice with PBS were added to 0.5 ml of a 1:10 dilution of VO-7 serum, incubated at room temperature for 1 h and then at 4°C for 7 h on a shaker. The mixture was centrifuged at 5000 g for 10 min, and the supernatant was collected. Absorption with uninfected Daudi cells was done similarly.

Chemicals. Cytosine arabinoside (hydrochloride) (Ara-C) was purchased from Sigma Chemical Co., St. Louis, Mo., or from Upjohn Co., Kalamazoo, Mich., U.S.A. Puromycin (dihydrochloride) was from Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A. Disodium phosphonoacetate (PA) was the kind gift of Dr K. Sato, Shionogi Pharmaceutical Co. Research Institute, Osaka, Japan.

RESULTS

Appearance of MA, EA and VCA in the superinfected and trypsin-treated Daudi cells

As shown in Fig. 1, the high background of membrane fluorescence in Daudi cells infected with P3HR-1 virus and treated with medium alone make the distinction between passively adsorbed and newly synthesized MA difficult. Trypsin treatment 2 h after infection removed most detectable MA from the cell surface and brought the background to the level of uninfected cells. The results agreed with our previous results with the superinfected NC-37 (C-6) cells (Sairenji & Hinuma, 1975) and another report (Dölken & Klein, 1976) in which the superinfected Raji cells were treated with papain.

Superinfected and trypsin-treated Daudi cells were examined for appearance of MA, EA and VCA (Fig. 1). The important increases in these antigens took place during the first 24 h after infection. The frequency of MA+ cells increased to a level of 10% 8 h after infection, decreased slightly thereafter but rose again between 14 and 18 h after infection. The reason for the appearance of the initial peak (8 h) of MA+ cells is unknown, though it has been consistently observed in repeated experiments. Both EA+ cells and VCA+ cells appeared simultaneously but 3 h later than that of MA+ cells. Frequency of EA+ cells and VCA+ cells increased gradually and reached a maximum level about 22 h after infection. The maximum value was 34% of EA+ cells and 23% of VCA+ cells. However, the maximum frequency of VCA+ cells was not always lower than EA+ cells but often the frequencies were similar, as described in an earlier report (Sairenji & Hinuma, 1973). The factor(s) responsible for the fluctuation is unclear.

The addition of 25 μg/ml of puromycin at the time of virus infection completely inhibited MA as well as EA and VCA synthesis (Fig. 2). This suggests that appearance of the antigens is due to de novo synthesis.
P3HR-1 virus infected and trypsin-treated Daudi cells were cultured in the presence of 20 µg/ml of Ara-C. Fig. 3 shows that Ara-C markedly inhibited the synthesis of both MA (Fig. 3a) and VCA (Fig. 3c) but not EA (Fig. 3b). The results suggest that not only VCA but also, in some cases, MA is dependent on DNA synthesis which is blocked by Ara-C (Gergely et al. 1976).

Since it was reported recently that PA specifically inhibits virus DNA synthesis in EBV-infected cells (Thorley-Lawson & Strominger, 1976; Yajima, Tanaka & Nonoyama, 1976), the effect of this drug on the synthesis of MA as well as EA and VCA was examined. As shown in Fig. 4, no effect was seen on EA synthesis (Fig. 4b) even at 200 µg/ml of PA. In contrast an inhibitory effect dependent on concentration of the drug was observed on both MA (Fig. 4a) and VCA (Fig. 4c) synthesis. In the presence of 200 µg/ml of PA, which suppressed the level of VCA+ cells (Fig. 4c) from about 40 % to 5 %, MA+ cells (Fig. 4a) were still detectable. This pattern of both PA-sensitive and insensitive fractions of MA synthesis was somewhat analogous to that in the experiments with Ara-C (Fig. 3a).

The immunofluorescent pattern of MA on cells cultured in the presence of Ara-C or PA differed from that obtained in the absence of the drugs, as shown in Fig. 5. The membrane fluorescence noted in superinfected Daudi cells without Ara-C or PA consisted more commonly of a nearly complete or full ring; less frequently, the fluorescence detected consisted
EBV-membrane antigen

Fig. 2. Appearance of EBV-associated MA (●), EA (○) and VCA (▲) in the P3HR-1 virus superinfected Daudi cells, in presence (----) or absence (-----) of puromycin (25 μg/ml).

Fig. 3. Effect of Ara-C (20 μg/ml) on appearance of EBV-associated (a) MA, (b) EA and (c) VCA in the P3HR-1 virus superinfected Daudi cells. ——, Without Ara-C; ----, with Ara-C.

Fig. 4. Effect of various concentrations of PA on appearance of EBV-associated (a) MA, (b) EA and (c) VCA in the P3HR-1 virus superinfected Daudi cells. ○—○, No PA; ●—●, 25 μg/ml; △—△, 50 μg/ml; ▲—▲, 100 μg/ml; ×—×, 200 μg/ml PA.

of several dots on the cell membrane. In the presence of Ara-C or PA, there were cells with partial rings or dots, but none with a full ring. Since there was a possibility that absence of a full ring MA may reflect the PA or Ara-C sensitive part of MA synthesis, the effect of PA on the appearance of the full ring MA was examined. As shown in Fig. 6, the appearance of a full ring MA was progressively reduced by increasing concentrations of PA until at 200 μg/ml it was completely inhibited. Hence, the full ring MA appeared to be a PA sensitive late product of the EBV genome.
Fig. 5. Indirect membrane immunofluorescent stains of the (a) P3HR-1 virus superinfected Daudi cells and (b) superinfected and Ara-C treated Daudi cells 24 h after infection.

Absorption test of MA reactive serum for differentiation of two MA components

If the Ara-C sensitive component of MA in the P3HR-1 virus superinfected Daudi cells is antigenically distinct from the Ara-C insensitive one of the MA, an appropriate MA-reactive serum could be differentially absorbed with the respective MA components. To test this possibility the presumably polyvalent serum, VO-7, was absorbed with one of three
EBV-membrane antigen

Fig. 6. Effect of various concentrations of PA on appearance of the full ring MA in the P3HR-1 virus superinfected Daudi cells in the same experiment as shown in Fig. 4. ○--○, No PA; ○--○, 25 μg/ml; △--△, 50 μg/ml; ▲--▲, 100 μg/ml; ×--×, 200 μg/ml.

Fig. 7. MA reactivity of the VO-7 serum against (a) Superinf·Daudi cells and (b) Superinf·Ara-C·Daudi cells before (○--○) and after absorption with the non-superinfected (●--●), the superinfected (▲--▲) or the superinfected and Ara-C treated (△--△) Daudi cells. Both absorbent cells and target cells were used at 24 h post infection.

Daudi cell preparations; the superinfected and Ara-C treated (Superinf·Ara-C·Daudi), the superinfected but not Ara-C treated (Superinf·Daudi) and the unsuperinfected and untreated (Daudi) cells. Each of these absorbed sera was examined for MA reactivity against the Superinf·Ara-C·Daudi and Superinf·Daudi. All infected cell preparations for either absorption or target were used 24 h after superinfection. Fig. 7 summarizes the results. The MA reactivity of the serum absorbed with Daudi cells was not significantly different from the unabsorbed serum for the Superinf·Daudi (Fig. 7a) and Superinf·Ara-C·Daudi (Fig. 7b) targets. The reactivity of the serum against the Superinf·Daudi target cells (Fig. 7a) was greatly reduced by the absorption with the Superinf·Daudi but only slightly with the Superinf·Ara-C·Daudi. In turn, the reactivity of the same serum against the Superinf·Ara-C·Daudi target cells (Fig. 7b) was reduced more by absorption with the Superinf·Daudi than with the Superinf·Daudi. However, it is notable that the Superinf·Daudi cells also possess the antigen shared with that of the Superinf·Ara-C·Daudi cells, because the reactivity of serum to the latter cells was reduced by absorption with the former cells to a certain extent (Fig. 7b).

In summary, the evidence indicates that the two antigenically different components of MA, Ara-C sensitive and Ara-C insensitive antigens, were found on the Superinf·Daudi cells. On the other hand, on the Superinf·Ara-C·Daudi cells, the Ara-C insensitive MA and only a small quantity, if any, of the Ara-C sensitive component were detected.
DISCUSSION

The present (Fig. 1) and previous (Sairenji & Hinuma, 1975; Dölken & Klein, 1976) studies clearly indicated that trypsin or papain treatment of cells immediately after super-infection made possible the study of the appearance of de novo synthesized MA without disturbing adsorbed virus, because the enzyme treatment reduced MA positive adsorbed materials to background level.

One component of MA in Daudi cells infected with P3HR-1 virus in the presence of Ara-C or PA was ‘early’ by definition because it appeared without virus DNA synthesis, which was blocked by the inhibitors (Gergely et al. 1971; Yajima et al. 1976). This MA may correspond to the early MA' (EMA) synthesized in the superinfected Raji cells in the presence of Ara-C (Ernberg et al. 1974). The existence of another component of MA on the superinfected Daudi cells in the absence of DNA inhibitors was suggested by a marked reduction in the frequency of MA+ cells in the cultures with these inhibitors (Fig. 3a and 4a). Therefore, this component can be referred to as late MA (LMA) and corresponds to the LMA present in the VCA+ cells in producer cell lines (Ernberg et al. 1974). The difference in antigenic specificity between EMA and LMA was demonstrated by the differential absorption of an MA reactive antiserum (Fig. 7). The antigenic distinction further strengthens the possibility that the two components of MA correspond to those observed by Ernberg et al. (1974).

The decrease in the frequency of MA+ cells in superinfected Daudi cells in the presence of DNA inhibitors (Fig. 3a and 4a) suggests that a large portion of the cells in the superinfected cultures are LMA+ EMA− on their surface. This concept is supported in part by the observation that the superinfected cells without Ara-C absorbed less EMA antibody than did the Ara-C treated cells (Fig. 7). However, synthesis of EMA may have taken place prior to synthesis of LMA on the same cells, but the EMA was masked or modified by LMA generated in the same topographic area of the cell surface. It is not known whether LMA is synthesized in any superinfected cell lines other than Daudi, e.g. Raji or NC-37, since Ara-C treatment did not significantly reduce the frequency of MA+ cells in these cultures (Sairenji & Hinuma, 1975; Dölken & Klein, 1976). This suggests that almost all MA+ cells in these superinfected cell lines may exhibit both EMA+ and LMA+ or only EMA+ on their surface. The present and other findings indicate that the Daudi cell line possesses a relatively high permissiveness for expression of the EBV genome when compared to other non-producer cell lines such as NC-37 (C-6) and possibly Raji. The following points are relevant: (1) When the same amount of EBV (P3HR-1 strain) was inoculated into the same number of cells, the frequency of EA+ cells (Sairenji & Hinuma, 1973) or MA+ cells (T. Sairenji & Y. Hinuma, unpublished data) in Daudi cells was about two times higher than that in NC-37 (C-6) cells, even though the virus adsorption capacity in the two cell lines was almost equal (Sairenji & Hinuma, 1973). (2) Similarly, VCA was produced in a much higher proportion of Daudi cells than in NC-37 (C-6) cells (Sairenji & Hinuma, 1973); this high level of VCA production in Daudi cells was reproduced in the present studies. (3) An amount of LMA, as represented by higher incidence of LMA+ cells, exceeded that of EMA+ cells, in Daudi cells but not in other superinfected cell lines (Sairenji & Hinuma, 1975; Dölken & Klein, 1976). (4) Production of matured virions was consistently observed in the superinfected Daudi cells by electron microscopy (K. Miyamoto & Y. Hinuma, unpublished data) although recently Yajima & Nonoyama (1976) reported production of virions in superinfected Raji cells in phosphate free-medium.
EBV-membrane antigen

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