Comparison of Herpes Simplex Virus Type 1 DNA Replication and Virus Production in Murine Bone Marrow-derived and Resident Peritoneal Macrophages

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SUMMARY

The mechanism of resistance of murine macrophages (Mφ) to infection by herpes simplex virus type 1 (HSV-1) was examined. Infection of bone marrow-derived Mφ (BMDMφ) and resident peritoneal Mφ (Res-Mφ) was compared with infection of permissive Vero cells. In contrast to HSV-1 infection in Vero cells, no infectious virus was produced from either Mφ cell type. However, marked cytopathic effect (c.p.e.) was evident in BMDMφ at 48 h post-infection, while there was no c.p.e. at any time post-infection in the Res-Mφ. Cloned EcoRI subgenomic fragments representing the entire HSV-1 genome were used as probes in DNA : DNA hybridization experiments to determine the viral genome content in the infected cell types. In Res-Mφ, HSV-1 DNA was present at early times post-infection but declined rapidly. In BMDMφ, the virus genome was always detected and increased with time after infection. The results suggest that Res-Mφ restrict HSV-1 production at a point prior to viral DNA synthesis, whereas the block in HSV production in BMDMφ occurs at a later stage in the viral replicative cycle.

The ability of macrophages (Mφ) to support virus replication varies markedly with the source of Mφ and the type of virus (Morahan et al., 1985; Mogensen, 1979). In general, Mφ are non-permissive for herpes simplex virus (HSV) replication, but the degree of non-permissiveness can be influenced by the genetic strain of animal used, the virus strain, the tissue source of Mφ and numerous other factors (for review, see Morahan, 1984; Mogensen, 1979). Stevens & Cook (1971) reported that HSV DNA is synthesized and empty capsids are present in Mφ that are unable to support production of infectious virus. However, it is not clear whether the block in virus production is at a point beyond viral DNA synthesis or whether aberrant viral DNA replication is responsible for the restriction of infectious viral growth.

In order to define better the mechanism of restriction in Mφ for HSV-1 replication at the molecular level, we have performed a comparative study of HSV-1 replication in murine bone marrow-derived Mφ (BMDMφ), murine resident peritoneal Mφ (Res-Mφ) and in the permissive monkey kidney (Vero) cells. BMDMφ provide a convenient source for obtaining large quantities of pure Mφ (Stewart, 1981). However, the developmental relationship of BMDMφ to other Mφ such as peritoneal Res-Mφ, that serve as a prototype for tissue Mφ, is not clear (Morahan, 1981). We used cloned EcoRI subgenomic fragments of the HSV-1 genome (kindly provided by Dr R. Sandri-Goldin) as probes in hybridization experiments to determine if the entire HSV-1 genome is present in BMDMφ and Res-Mφ if it is, whether the amount of viral DNA in these cells increased with time post-infection.

The growth of total infectious HSV-1 (KOS) particles in the three cell types is shown in Fig. 1. There was extensive viral replication in Vero cells, with marked cytopathic effect (c.p.e.) by 48 h post-infection. In contrast, there was no increase in virus titre, even by 48 h, when BMDMφ

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Fig. 1. HSV-1 growth kinetics and c.p.e. in Vero, BMDMϕ and Res-Mϕ cells. Res-Mϕ from female CD-1 mice (Charles River Laboratories) were harvested in phosphate-buffered saline containing 2 U/ml heparin, and allowed to attach to a plastic surface for 2 h at 37 °C. Non-adherent cells were removed by washing and the resulting Mϕ were incubated in maintenance medium (MM, MEM with Earle's balanced salts and 2% foetal calf serum) for 18 h at 37 °C before infection, when > 95% of the cells were spread Mϕ by morphological and phagocytic activity criteria. BMDMϕ were prepared by the method of Stewart (1981), which is now the standard method for preparing BMDMϕ in liquid culture. Briefly, BM cells which contained haemopoietic stem cells were obtained by flushing aseptically isolated femurs, and were incubated for 7 days in alpha-MEM containing 10% foetal cell serum, 10% horse serum, 10% L-929 cell-conditioned medium (a source of granulocyte-monocyte colony stimulating factor), 2 mm-glutamine, 25 mm-HEPES buffer and the antibiotics, penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml). At this time, essentially all of the adherent cells are very large well spread Mϕ by morphological, esterase staining and phogocytic activity criteria. Vero cells were cultured in MM at 37 °C overnight before infection. At the time of infection, parallel cultures of all cell types were counted by the Pronase-cetrimide procedure of Stewart (1981). Cell numbers were $2.1 \times 10^5$ cells per 16 mm diam. well for Res-Mϕ, $2.9 \times 10^5$ cells/well for BMDMϕ and $1.9 \times 10^5$ cells/well for Vero cells. Cells were infected with HSV-1 (KOS) at a m.o.i. of 5 (based on the amount of virus added for adsorption), and virus was adsorbed for 1 h at 37 °C. Unadsorbed virus was frozen for titration. Infected cells were washed three times with 0.5% gelatin, 5% lactalbumin hydrolysate, and Hanks' BSS and incubated in MM. At the times indicated, c.p.e. was recorded and the cells and supernatant fluid were harvested by scraping, and frozen at −70 °C for plaque titration of total virus yield on Vero cells (Morahan et al., 1977). –, No detectable c.p.e.; ++, 25 to 50% c.p.e.; +++, 50 to 75% c.p.e.; ++++, 75 to 100% c.p.e. •, Vero cells; ○, BMDMϕ; ▲, Res-Mϕ.

were infected with HSV-1 under the same conditions. There was, however, significant c.p.e. in these cells, which increased from 24 to 48 h post-infection. This suggests that some viral gene expression and possibly viral replicative events occurred in BMDMϕ without the final production of infectious virus. In contrast, the titre of HSV decreased in Res-Mϕ following the identical infection procedure, and there was no evidence of c.p.e. at any time.

The marked difference in the response of each cell type to viral infection led us to examine the viral DNA content of these cells. DNA from infected and uninfected cells was isolated, purified and immobilized on nitrocellulose filters as dot blots. The filters were then hybridized to plasmids containing the EcoRI fragments of the HSV-1 (KOS) genome. The construction and analysis of these chimeric plasmids has been described by Goldin et al. (1981), and the details of the hybridization methodology used in this manuscript are described in the legends to the figures. For dot-blot analysis, the HSV-1 EcoRI fragments D, G, N, F, M, O, A and I were used as probes. Collectively, these fragments represent about 65% of the entire HSV-1 genome. The
Fig. 2. Infected cell DNA dot blots hybridized with EcoRI fragments of HSV-1. Cells were plated and infected as described in Fig. 1. The cells were counted, centrifuged and frozen as pellets at −70 °C. DNA was extracted from 5 × 10⁶ cells suspended in 100 μl 10 mM-Tris-HCl, 1 mM-EDTA (TE) pH 7.5. Nine vol. 50 mM-EDTA, 0.5% SDS were added and the cells were digested with 100 μg/ml proteinase K (Sigma) for 3 h at 37 °C. DNA was extracted exhaustively with phenol and chloroform, digested with 100 μg/ml pancreatic RNase A (Sigma) for 2 h at 37 °C and precipitated in ethanol following further phenol:chloroform extraction. The A₂₆₀/A₂₈₀ ratio for each sample was 1.8. The DNA was sheared, denatured in NaOH, neutralized with HCl and spotted (2 μg) on BA-85 nitrocellulose filters (Schleicher & Schuell) according to Leary et al. (1983). Filters were stored over CaSO₄ at 4 °C until use. Plasmids containing EcoRI fragments of HSV-1 (KOS) (Goldin et al., 1981) were purified by CsCl-ethidium bromide centrifugation and were labelled with ³²P-dCTP by nick-translation (Rigby et al., 1977) using New England Nuclear kit number 004B and following the manufacturer's directions. Specific activities obtained ranged from 1 × 10⁹ to 5 × 10⁷ c.p.m./μg DNA. Prehybridization and hybridization conditions were essentially those of Wahl et al. (1979) as modified by Leafy et al. (1983). Probe concentration was 10 ng/ml, prehybridization was for 1 h and hybridization (in 50% formamide) was for 18 h at 41 °C. The filters were washed twice in 2 × SSC with 0.1% SDS at room temperature for 2 min; twice in 0.2 × SSC, 0.1% SDS at room temperature for 2 min; twice in 0.1 × SSC, 0.1% SDS at 50 °C for 15 min; finally twice with 2 × SSC, 0.1% SDS, at room temperature for 1 min. The filters were air-dried and exposed to X-ray film with intensifying screen for 4 to 72 h depending upon the specific activity of the probe. Each filter contained 2 μg DNA from HSV-1-infected BMDMφ, Vero and Res-Mφ harvested at 24 and 48 h post-infection and mock-infected cells (U) harvested at 24 h post-infection. Control HSV-1 DNA was purified virion DNA (Sandri-Goldin et al., 1981) spotted from left to right as 5000, 500 and 50 genome equivalents/cell in 2 μg herring sperm (Sigma) carrier DNA, except for the EcoRI D filter (5000, 5000 and 500). The filters were hybridized with plasmids containing EcoRI fragments A, G, D and O (Goldin et al., 1981) as labelled.
Short communication

To determine if the absence of viral DNA in Res-Mφ was due to the inability of these cells to take up virus, the identical dot-blot analysis was performed on HSV-1-infected Vero and Res-Mφ cell DNA extracted from cells harvested at 2 h post-infection (when only input DNA is present), using the EcoRI A fragment as a probe (Fig. 3). The cells were washed rigorously after the adsorption period. If virus had merely adsorbed but not penetrated Res-Mφ, the amount of radioactivity should be less than that in the Vero cell dots. However, both Vero cells and Res-Mφ contained approximately equal amounts of EcoRI fragment A at 2 h post-infection. This suggests that the absence of HSV-1 DNA at later times after infection in Res-Mφ is not due to the inability of HSV-1 to enter Res-Mφ, but may be due to a lack of viral DNA replication in these cells. Whether this event is mediated through defective synthesis or regulation of α or β genes remains to be studied.

Analysis for the presence of EcoRI fragments JK, EK, H and L was determined by Southern blot hybridization, because these portions of the HSV genome have been reported to contain sequences that cross-hybridize with cellular sequences (Peden et al., 1982; Puga et al., 1982; R. Sandri-Goldin, personal communication). These potentially cross-hybridizing fragments were used to determine if the remaining portions of the HSV genome were present on HSV-1-infected BMDMφ. Each fragment was present in both infected Vero cells and BMDMφ at the expected location based on their sizes (Goldin et al., 1981) (Fig. 4). Qualitatively, there was considerably more viral DNA in Vero cells than in BMDMφ, but clearly the entire HSV-1 genome was present in BMDMφ at 24 h post-infection. These data suggest that the block in HSV-1 production in BMDMφ occurs at a point beyond viral DNA synthesis.

Fragments JK and EK, which encompass the joint region and can exist in multiple forms in a population of HSV-1 genomes, appeared as more than one band, as expected, in both infected Vero cell and BMDMφ DNA. Some Vero cellular sequences also may have hybridized to the probes, sequences which were not present in the uninfected BMDMφ DNA EcoRI.

The present data clearly establish that sequences spanning the entire HSV-1 genome are present in infected BMDMφ, and that there is an increase after infection, suggestive of true viral DNA replication. This is in striking contrast to Res-Mφ, where HSV-1 DNA appeared to enter the cell as efficiently as in Vero and BMDMφ, but did not replicate. Due to the large size of the EcoRI fragments used, it is still possible that the block of HSV-1 production in BMDMφ is the result of small changes occurring during DNA replication that would not be detected by our
Fig. 4. Southern blots of Vero and BMD-Mφ infected cell DNA hybridized with HSV-1 EcoRI H, EK, L and JK fragments. HSV-1-infected Vero cells and BMDMφ, and mock-infected BMDMφ were harvested at 24 h post-infection and DNA was prepared as described in Fig. 1 and 2. Five μg of each cell DNA and 10, 5 or 0.5 ng of each plasmid DNA (in 3 μg of herring sperm DNA as carrier) were digested to completion with 25 h units of EcoRI (Bethesda Research Laboratories) for 5 h at 37 °C. The digested DNA was electrophoresed on agarose gels as described by Alwine et al. (1980), depurinated according to Wahl et al. (1979), and transferred to nitrocellulose sheets after denaturation, as described by Southern (1975) and modified by Thomas (1980). The migration distance is slightly different in each gel because, due to the large differences in sizes of the fragments, the gels were run for different lengths of times. Nick translation, prehybridization, hybridization and washing were performed exactly as in Fig. 2. Lane 1, 5 μg mock-infected BMDMφ DNA; lane 2, 5 μg infected BMDMφ DNA; lane 3, 5 μg infected Vero cell DNA; lane 4, blank; lanes 5 to 7, 0.5, 5 and 10 ng pBR325 plasmid DNA respectively in 3 μg herring sperm DNA. The location of the pBR325 specific plasmid DNA with the specific HSV-1 EcoRI fragment used on each filter is indicated on the right. The sizes of the DNA fragments are: JK, 17.7 kb; H, 14.8 kb; EK, 13.9 kb; pBR325, 5.4 kb; L, 5.2 kb.
experimental analysis. The fact that the EcoRI fragments were present in approximately equal amounts in BMDMφ, however, provides indirect evidence against this (Fig. 2). The mechanism involved in the non-permissiveness of the BMDMφ for HSV-1 may involve subtle changes in the viral DNA, especially in regulatory regions for transcription of late B or V gene products. However, sufficient viral genes were expressed to cause cell death in BMDMφ, which was not the case with Res-Mφ.

It is not yet clear whether there is abortive viral replication in all of the BMDMφ, or whether only a subpopulation of cells is productively infected. Experiments employing in situ hybridization on infected cells will help resolve this issue. The present investigation clearly documents heterogeneity in the extent of HSV-1 replication in Mφ. The origin of the diversity in Mφ permissiveness for HSV remains to be defined. There have been reports that HSV replicates more efficiently in macrophages that have differentiated from monocytes by culture in vitro for several days (Linnanvuori & Hovi, 1983). Thus, permissiveness for HSV may be an indicator of the state of differentiation or maturation of macrophages. Obviously, BMDMφ differ from the prototype peritoneal Res-Mφ in resistance to HSV. Our laboratory has recently demonstrated that BMDMφ also differ in other respects, such as ectoenzymes and polyamines, and appear to be at a less differentiated state than peritoneal Res-Mφ (Dempsey et al., 1984). Interestingly, data obtained on cytomegalovirus growth in embryonal carcinoma cells have shown that herpesvirus replicated more efficiently in the more differentiated cells (Dutko & Oldstone, 1981; Gonczol et al., 1984). Experiments are now underway in this laboratory to determine the status of HSV DNA in infected peritoneal Mφ at different stages of activation (Morahan et al., 1980).

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