Persistent Cyclic Herpes Simplex Virus Infection In Vitro

IV. Changes in the Severity of the Infections in the Presence of Antibody

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ABSTRACT

HAMPAR, BERGE (National Institute of Dental Research, Bethesda, Md.). Persistent cyclic herpes simplex virus infection in vitro. IV. Changes in the severity of the infections in the presence of antibody. *J. Bacteriol.* 92:1741-1747. 1966.—The severity of persistent herpes simplex virus (HSV) infections maintained in the presence of viral antibody (AB) changed with time. These changes could be divided into three distinct phases during which the severity of the infections were maintained at low (phase 1), high (phase 2), and intermediate (phase 3) levels. Paralleling these changes was the appearance in the cultures of a new HSV variant which induced the formation of giant cells. The changes in the severity of the infections were attributable to three factors. The first was the presence in the cultures of virus-resistant cells. The second was the suppressive effects due to virus neutralization by the AB. The third was the inherent properties of each HSV variant which determined the amount of virus remaining in an infectious form in the presence of AB. Finally, the period of "exacerbation" in vitro (phase 2) was compared with recurrent episodes in human herpes infection.

The apparent persistence of herpes simplex virus (HSV) in humans has stimulated extensive research to determine the mechanism by which the virus intermittently becomes clinically evident, although relatively constant levels of humoral antibody are continuously maintained (12). Much of this work has been carried out with persistent HSV infections established in vitro. In most of these cases, however, the cells employed were all susceptible to the cytopathic effects (CPE) of HSV, and the maintenance of the infections required specific supportive measures to avoid complete destruction of the cultures. These supportive measures included the addition of viral antibody to the medium (2, 9, 10, 16, 17), the maintenance of the cultures at suboptimal temperatures (1), the use of medium deficient in certain nutrients (13), the presence of a second virus to stimulate interferon production (3), and the addition of interferon to the medium (3). In contrast to the above systems, the persistent HSV infections established in the MAL line of Chinese hamster cells required no external supportive measures for their maintenance (7). These persistent infections were characterized by

cycles of virus synthesis and cell destruction, followed by regrowth of the cells (5-7).

This report describes modifications which occurred in the properties of persistent MAL cell infections maintained in the presence of HSV antibody (AB). These modifications resulted in changes in the severity of the infections, and were paralleled by the appearance in the cultures of a new HSV variant which induced the formation of giant cells. Evidence is presented that the effects of AB, when combined with the properties of the new HSV variant, resulted in a short-term "exacerbation" of the infections which was characterized by a dramatic increase in the virus titers and CPE.

MATERIALS AND METHODS

Cell cultures and virus. The HeLa cells, persistently infected MAL cells, tissue culture techniques, and media employed have been described (5-7). Primary rabbit kidney (PRK) cells were maintained in Eagle's medium supplemented with 0.5% lactalbumin hydrolysate and 10% calf serum plus antibiotics (ECS). The virus used for initiating persistent infections was the SAE strain of HSV (8) which had been passaged over 100 times in HeLa cells. The titration of HSV in

HeLa cells, and the recording of virus-induced CPE, have been described (7).

Virus titrations. Virus was titered by the tube dilution method ($TCID_{50}$) (7) in HeLa, MAL, and PRK cells, and as plaque-forming units (PFU) in MAL and PRK cells. No significant differences were noted when the same virus was assayed by these two methods. Further, no significant differences were noted when the same virus was assayed in the HeLa, MAL, and PRK cells.

Plaque purification and isolation of HSV variants. The virus from the medium of persistently infected cultures was plaque-purified in MAL and PRK cells. Approximately 10 PFU of virus were inoculated per culture. The cultures were incubated for 2 hr at 37 C. In the case of the MAL cultures, the cells were refed with medium containing 2% anti-HSV serum. After 3 to 4 days, the medium was removed, and the cultures were overlaid with medium containing 1% agar (Noble agar, Difco). In the case of the PRK cultures, the plaques were allowed to develop under an agar overlay consisting of ECS containing 1% agar and neutral red (1:50,000). The virus plaques in the MAL and PRK cultures were located microscopically, and they were classified as being either nonproliferative (NP) or giant cells (GC) types (4, 11). The plaques were removed with a capillary pipette and were transferred to either MAL or PRK cultures for virus propagation. As controls, areas not bearing plaques were isolated and inoculated into either MAL or PRK cultures. All controls were negative.

Preparation of viral antibody. New Zealand white rabbits (NIH strain) were employed for the production of AB. After corneal scarification of one eye of each rabbit under sodium pentothal anesthesia, HSV was applied with a cotton swab. The SAE strain of HSV produced no encephalitogenic effects in more than 80 animals inoculated. The animals were observed during the succeeding 5 days to ensure that a positive herpetic lesion developed. After a further 3-week period, the animals received 6 weekly intravenous injections of 10^6 to 10^7 tissue culture infective doses ($TCID_{50}$) of virus. The animals were then bled by cardiac puncture, followed by alternating weekly inoculations of virus and bleeding for 20 to 30 weeks. The sera were separated, pooled, and heat-inactivated at 56 C for 30 min. The AB used here was a single pool obtained from eight animals. The AB titers were calculated as that dilution which affected a 50% reduction in the number of PFU, when equal volumes of virus containing 10^4 PFU were reacted with AB dilutions for 30 min at 37 C. The titers so determined ranged from 1:160 to 1:180.

RESULTS

Severity of persistent infections maintained with AB. Antibody at a final concentration of 2.5% was added to the cultures immediately after the initiation of persistent infections (7). Parallel untreated cultures or cultures containing 2.5% normal rabbit serum served as controls. No differences in results were evident in the two types of control cultures. The severity of the infections

was defined as the percentage of the total cell population showing evidence of virus-induced CPE. In both the AB-treated and control cultures, the degree of CPE paralleled the virus titers in the medium (5), and either of these parameters could be used to determine changes in the severity of the infections. Figure 1 shows the virus titers in the medium from one typical experiment. The titers labeled "No AB" were from parallel control cultures where the AB either was initially omitted or was removed after passage of the cells on days 25, 55, and 82. As shown in Fig. 1, the severity of the infections in the AB-treated cultures was divisible into three distinct phases. The first phase persisted until day 78, and was characterized by a low level of infection. The CPE during this phase in both the AB-treated and control cultures was of the nonproliferative (NP) type (4, 11), with the cells rounding up and essentially remaining mononuclear (Fig. 2A). The second phase lasted from days 78 to 118, and was characterized by a rapid increase in the severity of the infections to a high level. The CPE in the AB-treated and control cultures during phase 2 consisted of a mixture of the NP type and a new type characterized by the formation of multi-nucleated giant cells (GC; Fig. 2B). The appearance in the cultures of giant cells suggested that a new HSV variant was being produced. The third phase occurred subsequent to day 118, and was characterized by a reduction in the severity of the infections to an intermediate level. The CPE subsequent to day 118 in both the AB-treated and control cultures was only of the GC type. Virus isolation and plaque purification in PRK and MAL cells substantiated that the change in the appearance of the CPE in the persistently infected cultures was due to a transition in the virus being produced from a NP to a GC variant. The distinctive appearance of the CPE induced by the NP and GC viruses was retained after numerous passages in PRK, HeLa, or MAL cells, suggesting that these HSV variants were genetically different (11).

The results illustrated in Fig. 1 indicated a relationship between the severity of the infections in the AB-treated cultures and the virus type being produced. To study this further, control cultures were divided into two groups, depending on which HSV variant was being produced. Antibody at a final concentration of 2.5% was added to half the cultures in each group, and the virus titers in the medium were determined. In all cases, the same variant continued to be produced throughout the experimental period. The results are shown in Fig. 3 for cultures producing either the NP (Fig. 3A) or the GC (Fig. 3B) variant. As shown in this figure, the virus titers were signifi-

FIG. 1. Virus titers in the medium of persistently infected cultures maintained in the presence of AB. The cultures employed were 50th passage MAL cells infected with 10^6TCID_{50} of HSV. The titers labeled "No AB" represent parallel cultures from which the AB was either initially omitted, or was removed subsequent to passage of the cells on days 25, 55, and 82. The virus titers were determined in HeLa cells. N.D., no virus detected.

FIG. 2. Appearance of lesions produced by the NP variant (A) and GC variant (B) in MAL cells in the presence of AB. Giemsa stain. $\times 63$.

cantly reduced over a 20- to 30-day period after addition of the AB. In the AB-treated cultures producing the NP variant (Fig. 3A), the titers were ultimately reduced to low levels, whereas

the titers in the cultures producing the GC variant (Fig. 3B) remained at intermediate levels. In both cases, the patterns indicated cycles of virus synthesis. These results support the finding

FIG. 3. Comparison of virus titers in the medium after the addition of AB to persistently infected cultures producing NP virus (A) and GC virus (B). The virus titers were determined in HeLa cells. N.D., no virus detected.

illustrated in Fig. 1, that a relationship existed between the severity of the infections in the AB-treated cultures and the virus type being produced.

Localization of infectious virus in the medium of the AB-treated cultures. The absence of significant amounts of infectious virus in the medium of the AB-treated cultures producing the NP variant was probably due to the neutralization of this virus by the AB, with a concomitant reduction in the severity of the infections. The inability of the AB to eliminate infectious virus from the medium when the GC variant was being produced suggests two possibilities. First, the GC variant may have been more resistant than the NP variant to neutralization by the AB employed, or, second, more of the GC virus than NP virus in the medium may have been in a form inaccessi-

ble to the action of the AB. Studies were undertaken to test these two possibilities. The rationale for these experiments was based on observations previously reported (5), that the virus in the medium from persistently infected cultures was separable into two fractions after centrifugation at $800 \times g$ for 10 min. The virus in the supernatant fraction was susceptible to relatively complete neutralization with AB. In contrast, only 90% of the virus associated with the sedimentable fraction was in a form accessible to the action of the AB, with the remaining 10% of the virus being resistant unless the cellular material was first disrupted by sonic oscillation. Studies were carried out, therefore, to determine with what fraction of the medium in the AB-treated cultures the infectious virus was associated. The medium from AB-treated cultures was collected and fractionated at $800 \times g$ for 10 min (5). The virus titers in the complete medium (cells plus fluid), the supernatant fractions (extracellular fluid), and the sedimentable fractions (cells) were determined. The results are shown in Fig. 4 for cultures producing either the NP (Fig. 4A) or the GC (Fig. 4B) variant. As shown in this figure, the infectious virus in the medium with both variants was associated with the sedimentable fractions, whereas the supernatant fractions contained little or no infectious virus. Therefore, the infectious virus in the medium of the AB-treated persistently infected cultures was associated with cellular material which sedimented at $800 \times g$, and was probably inaccessible to the action of the AB.

These results suggest that the higher titers noted in the AB-treated cultures when the GC virus was being produced were due to the greater tendency of this variant to remain associated with cellular material which sedimented at $800 \times g$. To study this, HeLa, PRK, and MAL cells were infected with plaque-purified NP or GC virus at a virus-cell ratio of one. At the time of maximal CPE (16 to 24 hr), the media were collected and were fractionated by centrifugation at $800 \times g$ for 10 min (5). The virus titers in the complete medium and in each of the fractions were determined. Similarly, the susceptibility of the virus in the supernatant and sedimentable fractions to neutralization when treated with AB at a final concentration of 10% for 30 min at 37 C was determined. The results illustrated in Table 1 with virus grown in MAL cells were the same regardless of which cell line was employed. The virus titer in the complete medium (A) with the NP variant was consistently higher than that found with the GC variant, regardless of which cells were used for virus propagation. This difference, however, was not noted when the

FIG. 4. Localization of virus in complete medium and the extracellular and sedimentable fractions of persistently infected cultures producing NP virus (A) and GC virus (B). The virus titers were determined in HeLa cells. N.D., no virus detected.

virus isolated from the medium of the persistently infected cultures was assayed. As shown in Table 1, almost all the NP virus was present in the supernatant fraction (B), and only 1 to 10% remained associated with the sediment (F). In contrast, almost all of the GC virus was present in the sedimentable fraction (F), and only 1 to 10% remained associated with the supernatant fraction (B). The virus in the supernatant fractions represented cell-free virus or virus associated with small fragments of cell debris which did not sediment at $800 \times g$. These results suggest that the GC virus had a greater tendency than did the NP virus to remain associated with intact host cells or large fragments of host-cell material which were sedimentable at $800 \times g$. It is also apparent from Table 1 that, with both variants, over 99.9% of the virus in the supernatant fractions (B) was in a form accessible to neutralization by the AB (D). In contrast, only 90% of the NP and GC virus in the sediments (F) was accessible to the action of the AB (H), with the remaining 10% being resistant unless the cellular material was first disrupted by sonic oscillation (K). It can be concluded from this that both the NP and GC variants were equally susceptible to neutralization by the AB employed, providing the virus was in an accessible form. When the original titers (A) are compared with the residual titers after treatment with AB of the supernatant (D) and sedimentable (H) fractions, it is apparent that over 99% of the total NP virus present in the medium was in a form accessible to neutralization by the AB, in contrast to only 90% of the GC virus. Since the percentage of each variant susceptible to neutralization in the supernatant and

TABLE 1. Intra- and extracellular localization of nonproliferating (NP) and giant cell (GC) virus in the medium of MAL cell infected cultures

Fraction	Serum treatment	Virus titers (TCID ₅₀ /ml)	
		NP	GC
A. Complete medium	—	10 ^{7.5}	10 ^{5.5}
B. $800 \times g$ supernatant fraction	—	10 ^{7.5}	10 ^{4.0}
C. $800 \times g$ supernatant fraction	Normal ^a	10 ^{7.3}	10 ^{4.3}
D. $800 \times g$ supernatant fraction	Anti-HSV ^b	10 ^{3.0}	10 ^{0.6}
E. $800 \times g$ sediment (wash)	—	10 ^{4.0}	10 ^{2.5}
F. $800 \times g$ sediment	—	10 ^{6.0}	10 ^{5.3}
G. $800 \times g$ sediment	Normal	10 ^{6.3}	10 ^{5.5}
H. $800 \times g$ sediment	Anti-HSV	10 ^{5.0}	10 ^{4.6}
I. $800 \times g$ sediment (disrupted)	—	10 ^{6.3}	10 ^{5.5}
J. $800 \times g$ sediment	Normal	10 ^{6.3}	10 ^{5.3}
K. $800 \times g$ sediment	Anti-HSV	10 ^{3.0}	10 ^{2.0}

^a Treated with normal rabbit serum at a final concentration of 10% for 30 min at 37 C.
^b Treated with AB at a final concentration of 10% for 30 min at 37 C.

sedimentable fractions was the same, the difference noted in the residual titers after AB treatment was probably due to the greater tendency of the GC variant to remain associated with the cellular material which sedimented at $800 \times g$. Further, since the same results were observed regardless of the host cell employed, it would appear that the difference in the amount of virus associated with the supernatant and sedimentable fractions of the medium was an inherent property of each HSV variant.

DISCUSSION

The findings presented here demonstrate that, despite the continued presence of AB, changes occurred in the severity of the persistent MAL cell infections. The changes were divisible into three distinct phases, during which time the severity of the infections was maintained at a low (phase 1), high (phase 2), and intermediate (phase 3) level. To understand the mechanism of these changes, it is first necessary to consider some of the general characteristics of these persistent infections. Studies to be reported separately indicate that an equilibrium was maintained in the persistently infected cultures between the number of susceptible cells and the infectivity of the virus. At any one time during the cycle, only a portion of the total cell population was susceptible to virus (6). The susceptible cells were infected, and subsequently were eliminated due to CPE, whereas the resistant cells remained intact (6). These latter cells proliferated to replenish the cultures (6). The elimination of the susceptible cells and the selection of resistant cells resulted in continual changes in the properties of the cell population. Similarly, the infectivity of the virus being produced at any one time was somehow influenced by the cells in which the virus was propagated. Consequently, changes with time occurred in the properties of both the cells and the virus. The combined effects of these cellular and viral changes accounted for the following temporal relationships. The cells which were present at any time T were more susceptible to the virus produced in the same culture at any time $> T$ than to the virus produced at any time $< T$. Conversely, the virus produced at any time T was more infective for the cells which were present in the same culture at any time $< T$ than for the cells which were present at any time $> T$. The important point to stress is that the conversion from resistance to susceptibility was due to viral and not cellular changes. Consequently, the cells' resistance or susceptibility to the virus present at any one time was a stable property.

The changes noted in the severity of the infec-

tions in the AB-treated cultures can best be explained as follows. Regardless of which HSV variant was being produced, at least a few focal lesions were evident in the cultures. These infective foci could expand to reach the same size as those in the untreated cultures, since the cell-to-cell transmission of virus was not sensitive to inhibition by AB (14, 15). In both the AB-treated and control cultures, however, the focal lesions were ultimately terminated when all of the infected cells were completely surrounded by resistant cells (6). Therefore, the spread of the infections to distant areas depended on virus transmission through the medium (5). When AB was present, the extracellular transmission of virus was prevented, and the initiation of new infective centers depended on the cell-to-cell transmission of virus via the infectious cellular material in the medium (5). The properties of the NP variant were such that less than 1% of the total virus in the medium remained infectious in the presence of AB (Table 1). Because of the small amount of infectious virus, the number of infective centers was proportionately reduced, and the severity of the infections was maintained at a low level (phase 1). Similarly, the relative lack of infectious virus precluded the elimination of susceptible cells from the cultures. Therefore, during phase 1, the proportion of cells in the population susceptible to virus was maintained at a significantly higher level than in the parallel control cultures. After the appearance of the GC variant in the AB-treated cultures, approximately 10% of the total virus in the medium remained infectious (Table 1). This greater amount of virus plus the high proportion of susceptible cells allowed the infections to be rapidly disseminated throughout the cultures. Consequently, the severity of the infections increased to a high level, despite the continued presence of AB in the medium (phase 2). This period of "exacerbation" lasted for only one to two cycles, however, due to infection and elimination of susceptible cells by virus. The proportion of the cell population remaining susceptible was thus reduced to the same level as that in the control cultures producing the GC variant at the time of AB addition (Fig. 3B and 4B). This decrease in the number of susceptible cells was followed by a reduction in the severity of the infections to a low level (phase 3). Consequently, the factor limiting the severity of the infections when only the NP variant was being produced (phase 1) was the availability of infectious virus. During the period when only the GC variant was being produced (phase 3), the factor limiting the severity of the infections was the availability of

susceptible cells. The period of transition from NP to GC virus (phase 2) was the time when the shift from virus to cell dependence occurred.

The appearance of new HSV variants in persistent infections requiring AB for their maintenance has been described (9, 10). The findings here confirm and extend these observations by demonstrating that viral changes occurred in persistent HSV infections whose maintenance required neither AB nor any other external supportive measures. The sequence of events outlined above for persistent MAL cell infections in the presence of AB suggested that the changes noted in the severity of the infections after the appearance of the GC variant were partially dependent on fluctuations in the proportion of virus-resistant cells in the population. This requirement for resistant cells makes it improbable that similar changes would occur in persistent HSV infections where all of the cells were susceptible to virus (1, 3, 10, 13, 16, 17). If viral changes comparable to the NP and GC variants occurred in such infections, one would expect to see an increase in the severity of the infections, such as described in the MAL cell system during phases 1 and 3, since such a change could be accounted for by an increase in the amount of infectious virus available for disseminating the infection. However, phase 2, which was characterized by a short-term "exacerbation" in the MAL cell infections, would not be expected to occur in persistent infections whose maintenance required external supportive measures.

Finally, it is of interest to note the possible similarity between the period of "exacerbation" in these in vitro infections (phase 2) and the recurrent episodes in human herpes infection. If the persistent MAL cell infections should prove to be analogous to the human disease, the results noted here would support the hypothesis that the virus in humans is maintained in a "dynamic" or infectious state during periods of remission (14). The recurrent episodes, therefore, would depend on alterations in the cell-virus equilibrium which could be correlated with changes in the properties of the virus being produced. It is anticipated that further studies will help clarify this issue.

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