Resistance to herpes simplex virus type 1 and its latent infection of human T cell lymphotropic virus type I-transformed T cell lines of rabbits

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Fourteen T cell lines of rabbits were infected with herpes simplex virus type 1 (HSV-1) and examined for their susceptibility to lytic infection and ability to support virus replication. T cell lines of CD4\(^{+}\) phenotype were more vulnerable to lysis and supported higher levels of virus replication than those of other phenotypes. Cell lines of CD4\(^{+}\) and CD4\(^{+}\) phenotypes continued to proliferate, while remaining productively infected, for more than a month. These latently infected cell lines could be established by treatment with anti-HSV-1 serum and complement. Viral genes were only partially expressed and the CD8 membrane antigen was down-regulated. Infected cell lines, as well as peripheral blood lymphocytes, were shown to induce meningoencephalitis when inoculated intravenously into syngeneic hosts, suggesting a possible role for infected lymphocytes in HSV-1 transport in vivo.

Introduction

Herpes simplex virus type 1 (HSV-1) causes a number of diseases ranging in severity from fever blisters to life-threatening encephalitis. However, the most important feature of this virus is its capacity to remain latent and to induce recurrent infections. It is well established that the virus may remain latent in the neurons of sensory ganglia, but latency does not only occur in these cells. Lymphocytes have also been reported to become latently infected (Rinaldo et al., 1978; Hammer et al., 1981). The expression of viral genes is wholly or partially repressed in these cells, with a restricted range of viral genes transcribed during latency.

The genetic susceptibility of B\(^{ }\)Jas inbred rabbits to herpetic encephalitis, a recessively inherited disease involving multiple genes, has recently been reported (Seto et al., 1995). One of the unique findings in these diseased rabbits was lymphocytopenia associated with the appearance of interferon-gamma (IFN\(\gamma\)) in the serum at the onset of encephalitic seizures. Serum IFN\(\gamma\) was apparently produced by T lymphocytes that infiltrated encephalitic lesions, because no pathological changes indicating HSV-1 infection were observed in any organ other than the brain, and the level of IFN\(\gamma\) was much higher in the cerebrospinal fluid than in the serum (unpublished data). This finding suggested that T lymphocytes infiltrating brain lesions were resistant to HSV-1 infection and were stimulated to produce a large amount of IFN\(\gamma\).

This led us to carry out the present study, which was intended firstly to examine the susceptibility of various T cell lines to infection by HSV-1, and secondly to characterize the nature of the infection, i.e. cytocidal, persistent or latent. This experiment identified subsets of T lymphocytes which were more resistant to infection than others and facilitated the establishment of latently infected cell lines. Latently infected cell lines were analysed for expression of viral antigens and genes, and the aetiological significance of such lymphocytes in circulation was investigated with regard to virus dissemination and pathogenesis of herpetic encephalitis in B\(^{ }\)Jas rabbits. Whether or not the same is the case in normal lymphocytes in vitro as well as in vivo will be reported elsewhere; the present paper is confined mostly to human T cell lymphotropic virus type I (HTLV-I)-transformed T cells.

Methods

- **Rabbit.** Inbred rabbits of strain B\(^{ }\)Jas were used. This strain was originally derived from the B\(^{ }\)J strain from the Jackson Laboratory (Fox, 1975) and has been maintained in the Institute for Experimental Animals, Shiga University of Medical Science, Japan.
- **Virus.** A laboratory strain (KOS) and a clinical isolate (Imasono) of HSV-1 and a laboratory strain (333) of HSV-2 were propagated in Vero...
cells which were cultured in Eagle's minimum essential medium supplemented with 5% foetal calf serum (FCS). The virus stock was prepared by infecting Vero cells with the virus, freeze–thawing infected monolayers and centrifuging the lysate at 2000 r.p.m. for 20 min to eliminate cell debris. The virus stock was assayed for infectivity by the plaque method (Ohashi & Ozaki, 1981), dispensed in small vials and stored at −80 °C until use.

**T cell line.** HTLV-I-transformed T cell lines were obtained as described (Soto et al., 1987, 1988a, 1988b; Takahashi et al., 1991). These cell lines comprised nine helper T cell lines (B405, B408, B245, B664, B779, H446, H557, F648h, F711), two killer T cell lines (F742, KSH5b) and two T cell lines of immature phenotypes (B761.3, F647a). The herpesvirus ateler-transformed cell line, RL-5, was also included as a helper T cell line. These cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS, unless otherwise noted.

**Latently infected T cell lines.** HTLV-I-transformed T cell lines B761.3 and F742 were infected with HSV-1 at an m.o.i. of 1 p.f.u. per cell and established as latently infected cell lines. Briefly, infected cells on day 3 post-infection (p.i.) were treated at 37 °C for 1 h with rabbit anti-HSV-1 serum no. 04 (Ohashi & Ozaki, 1981) plus guinea-pig complement and then cultured in RPMI 1640 medium containing 10% FCS. The treatment was repeated at intervals of 1–3 weeks and non-producer cell lines B(3) and F(3) were established from B761.3 and F742, respectively. Producer cell line B(1), obtained after a single treatment of infected B761.3, was also used in the experiment. These cell lines were maintained in culture using RPMI 1640 medium containing 5% FCS and 5% normal rabbit serum. F(5) was treated at intervals with fresh anti-HSV-1 rabbit serum to reactivate cell growth.

**RT–PCR analysis of viral gene expression.** Total cellular RNA was extracted with guanidinium thiocyanate–phenol–chloroform (Chomczynski & Sacchi, 1987) and the RNA sample was treated with DNase I (RNase free; Pharmacia Biotech) as recommended by the supplier. A 5 μg aliquot of total RNA was reverse-transcribed in 20 μl of solution with the use of random primers, and a portion (1 μl) of the resulting cDNA solution was amplified by PCR. The primer pairs used were (i) for latency-associated transcript (LAT), 5′ primer GACAGC-AAAAATCCCTCGAG and 3′ primer ACAGAGGAAAAACATAAAGGG (Chen et al., 1995); (ii) for glycoprotein B (gB), 5′ primer ATCTCTCTCCGAGCCATATTCCACCTTT and 3′ primer AGAAGCCCCCATTTGCGAGGAT (Ramakrishnan et al., 1994); (iii) for DNA polymerase (pol), 5′ primer CAGTACGGCCCCGAGTTCG-GTGACCAG and 3′ primer GGCGTGCCATGAA 3′; and the second pair consisted of an identical 20 cycles at 96 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and 72 °C for 3 min. Amplified DNA products were subjected to electrophoretic analysis on 2% agarose gel. In addition, equivalent amounts (0.25 μg) of RNA samples without reverse transcription were also PCR-amplified with the same primer pairs as for cDNA to ensure that signals detected by RT–PCR were not due to contaminated DNA.

**Assay of susceptibility of T cell lines to HSV-1 infection.** A suspension of 2 × 10⁶ cells was divided into four tubes and sedimented by centrifugation. Three strains of virus were added, respectively, to the cell pellet in three of the tubes at an m.o.i. of 10 p.f.u. per cell; the remaining tube was left for uninfected control cells. After 1 h at 37 °C, cells were washed once, resuspended in 2 ml of the medium and dispensed into wells of 24-well microplates. Cultures were microscopically observed for 5 days and then half of the medium was replaced on day 3. On day 5, viable cells were determined by the trypan blue exclusion test.

**Assay for the replication of HSV-1 in PBL, splenocytes and T cell lines.** Peripheral blood lymphocytes (PBL) were isolated from heparinized blood by sedimentation in 1% gelatin, followed by lysis of residual erythrocytes in isotonic ammonium chloride solution. Splenocytes were obtained by teasing spleen with forceps and scissors, and erythrocytes in the resulting cell suspension were lysed in ammonium chloride solution. These isolated cells, fresh or stimulated in culture with phytohaemagglutinin (PHA) for 48 h, as well as T cell lines, were adjusted to a cell density of 10⁷ per ml in Hank's solution, infected at an m.o.i. of 1 with the KOS strain of HSV-1 for 1 h at 37 °C, washed three times with Hank's solution and resuspended in RPMI 1640 medium containing 10% FCS. One ml aliquots of 0·25 × 10⁶ or 1 × 10⁶/ml cell suspension were dispensed in six tubes, and five of these were incubated at 37 °C in an atmosphere of 5% CO₂ in air; one tube was immediately frozen at −80 °C. One tube daily was removed from incubation to be kept frozen until used in the virus assay. The medium was not changed during this incubation period. Assay for infective virus was carried out by the plaque method (Ohashi & Ozaki, 1981) on the supernatants of cell lysates, which were prepared by freeze–thawing and centrifugation at 2000 r.p.m. for 20 min.

**Fluorocytometric analysis of HSV-1 antigen.** Cells were washed once with PBS and allowed to react at 4 °C with FITC-labelled anti-HSV-1 rabbit antibody (Chemicon) diluted in PBS containing 0·1% sodium azide. Stained cells were washed three times and analysed with FACScan (Becton Dickinson).

**Inoculation of infected cells into rabbits and detection of inoculated cells and infective virus.** Infected cells for injection into rabbits were prepared by mixing cells with HSV-1 (KOS) at an m.o.i. of 1, allowing the mixture to stand at 37 °C for 1 h and then washing the cells three times. Rabbits were injected intravenously (i.v.) with 1 × 10⁸ infected cells and kept under observation for 1 month. Animals were killed 3 days p.i. or at the onset of seizures by injecting a lethal dose of pentobarbital and were then subjected to pathological, virological and molecular biological examinations. Small fragments of various tissues were immersed in 10% formalin and Hanks' solution, or immediately frozen. Formalin-fixed tissues were embedded in paraffin, and sections were stained with haematoxylin and eosin for routine histological examination. Tissues in Hanks' solution were frozen once, thawed, homogenized to produce a 10% suspension, and overlaid onto monolayers of Vero cells. After a 1 h incubation, culture medium was added and the culture was kept under observation for 1 week. The appearance of typical cytopathic effects was taken as an indication of infection. Detection of inoculated HTLV-I-transformed cells (B761.3) was carried out by amplifying the HTLV-1 cellular junction sequence using nested primers and two rounds of PCR (H. Yoshida, Y. Nagano, T. Isono & A. Sato, unpublished). The first primer pair was B761.3 primer (5′ CGTGGCTCTGACATAGAT 3′) and LTRp6 (5′ AGCCATAT-GCGTGGCATGAA 3′); and the second pair consisted of an identical B761.3 primer with LTR3A (5′ CTTTGACACTCTTGCTTGGA 3′). Both rounds consisted of 30 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min.

**Results**

**Susceptibility of various T cell lines to HSV infection**

Fourteen T cell lines were infected with HSV and their susceptibility to HSV infection was compared by counting...
Table 1. Effect of HSV infection on the viable cell number of various T cell lines in culture

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<thead>
<tr>
<th>T cell line</th>
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<td>KOS</td>
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<td><strong>CD4+8</strong> T cell lines with αβ TCR**</td>
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<tr>
<td>B405 (IFNγγ)†</td>
<td>8+</td>
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<tr>
<td>F648b (IFNγγ)</td>
<td>8</td>
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<tr>
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<tr>
<td>H556 (IFNγγ)</td>
<td>10</td>
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<td>B408 (nα)</td>
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<td>B779 (nα)</td>
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<td><strong>T cell lines of other phenotype</strong></td>
<td></td>
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<tr>
<td>KSH5b (CD4+8, γδ, IFNγγ)</td>
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<td>F647a (CD4+8, αβ, IFNγγ)</td>
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<td>B761.3 (CD4+8, αβ, IFNγγ)</td>
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<td>F742 (CD4+8, αβ, IFNγγ)</td>
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* From Isono et al. (1996). † Expressed as percentage of viable cell number. ‡ Also including CD4+8 cells. nα, Not determined.

Fig. 1. Virus replication in (a) seven T cell lines and (b) splenocytes and PBL. HTLV-I-transformed T cell lines, PHA-stimulated or unstimulated splenocytes and freshly isolated PBL were infected at an m.o.i. of 1 p.f.u. per cell and virus yield determined for days 1–5 p.i. Open symbols indicate CD4+8 cell lines. Splenocytes were cultured for 48 h before infection in the presence or absence of PHA.

Virus replication in various T cell lines

HSV replication in seven T cell lines was comparatively studied by infecting cells with the KOS strain and determining virus titre in the culture for 5 days p.i. (Fig. 1a). The three cell lines of the helper type showed similar changes in virus titre, which resembled those in splenocytes (Fig. 1b), i.e. initial increase followed by an abrupt decline as observed in PBL. This initial increase in virus titre indicated HSV replication in these T cell lines and the abrupt decline indicated the lysis of infected cells, as reported in studies on non-transformed lymphocytes (reviewed in Kirchner, 1982). These results, together with those in Table 1, demonstrate that the helper T cell lines were lytically infected with the KOS strain, leading to high yields of progeny virus.

Four other cell lines of different phenotypes also supported virus replication, but to different degrees, and the change with time in virus titre varied according to cell lines. Both F647a and KSH5b cell lines supported virus replication without a decline in virus titre for 5 days of culture, while both F742 and B761.3 supported much less, albeit almost constant, replication. The virus yield was the smallest in B761.3. These results, together with the increase in viable cell number observed in Table 1, indicated that these cells were permissive but hardly susceptible to lysis, at least for 5 days. All these cell lines were semi-permissive and were persistently infected with the KOS strain, establishing apparently stable relationships between cell growth and virus replication.

Expression of HSV-1 antigen in resistant cell lines infected with HSV-1

Sequential change of viral antigen expression was monitored by FACS analysis of two HSV-resistant cell lines, B761.3 and F742. The results are shown in Fig. 2. Most infected B761.3 cells remained negative for 1 week p.i. and the proportion of positive cells gradually increased thereafter, to more than a half at 3 weeks. Infectious virus was detected by plaque assay of the supernatant of these cultures (not shown). Most cells had died by 5 weeks, as estimated by a trypan blue exclusion test. As for the F742 cell line, more than half of the infected cells were positive by day 1 p.i. and this proportion increased gradually up to more than 90% at 6 weeks. The positive signal obtained at 24 h p.i. was considered to represent active synthesis of viral proteins and not to be due to remnants of infected cells.

viable cells on day 5 of culture. The results are shown in Table 1. T cell lines of CD4+8 phenotype, irrespective of IFNγ production, were more susceptible than those of other phenotypes including immature and killer T cell lines. Not only HTLV-I-transformed cells but also herpesvirus aleles-transformed cells of the helper phenotype were highly susceptible to HSV. Cell damage by the clinical isolate of HSV-1 was more manifest than that by the laboratory strains of HSV-1 and -2, and this was consistently observed in all cell lines examined. No cell damage was observed in KOS-infected immature and killer T cell lines, although the growth of these cells was slightly retarded compared to uninfected controls.

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Fig. 2. Sequential change of viral antigen expression in HSV-1-resistant cell lines in culture. Two HTLV-I-transformed T cell lines, F742 and B761.3, were infected at an m.o.i. of 1 p.f.u. per cell and analysed with FACS at the indicated times p.i. Cells were stained by FITC-labelled polyclonal anti-HSV-1 antibody. The abscissa represents relative fluorescence intensity and the ordinate the cell number.

Fig. 3. Fluorocytometric analysis of viral antigen expression in latently infected cell lines. Two HTLV-I-transformed cell lines, F742 and B761.3, were infected with HSV-1 and three latently infected cell lines were established. F(5) is derived from F742; B(1) and B(3) from B761.3. Cells were stained by FITC-labelled polyclonal anti-HSV-1 antibody. The abscissa represents relative fluorescence intensity and the ordinate the cell number.

of input virus, because cells infected and then stained immediately after were negative (not shown). Most cells had died by 8 weeks. These results are consistent with the data presented in Table 1 and Fig. 1. Both B761.3 and F742 cell lines were resistant to HSV-infection, but gradually yielded to virus lytic activity as virus expression increased.

Establishment of latently infected cell lines, limited expression of the viral genes and down-regulation of CD8 antigen in these cell lines

Repeated treatments of B761.3 and F742 with rabbit anti-HSV-1 serum and guinea-pig complement allowed us to obtain latently infected cell lines which differed in viral gene expression. FACS analysis of these cell lines is shown in Fig. 3. Both B(1) and B(3) cell lines, which were derived from B761.3 through treatment once and three times, respectively, contained few cells expressing viral antigens, whereas most cells of the F(5) cell line, derived from F742, expressed viral antigens. Presence of the virus genome in these cell lines was confirmed by PCR, and the expression of three viral genes, pol, gB and LAT, was examined by RT–PCR (Fig. 4). The results showed that pol was expressed in B(1) alone and that gB was expressed in B(1) and, to a much lesser extent, in B(3); gB was not expressed in F(5), although most cells of this cell line expressed viral antigens on the membrane, as detected by polyclonal fluorescent antibody assay. Expression of gB in B(1) and B(3), but not in F(5), was confirmed by a fluorescent antibody technique using a monoclonal anti-gB antibody (data not shown). In agreement with these findings, infectious virus was repeatedly detected for more than 6 months in B(1) but not in the others by both plaque assay of the culture supernatant and infectious centre assay. Virus was not induced in these cell lines by treatment with PHA (data not shown). B(1) and B(3) continued to proliferate when cultured in medium supplemented with fresh rabbit serum, and, when this serum was removed from the medium, consistently resulted in such gradual deterioration as observed for antibody-untreated cells. Inactivated serum could not replace fresh serum. Similarly cultured F(5) gradually ceased to proliferate and finally died even in the presence of fresh rabbit serum and required treatment at intervals with fresh anti-HSV-1 serum for maintenance in culture. These findings suggested that some heat-labile factor(s) or anti-HSV-1 antibody stimulated these cell lines to proliferate through contact with virus infection-associated component(s) on infected cells.

Fluorocytometric analysis of other antigens in these cell
HSV-1 infection of HTLV-I-transformed T cells

RNA
DNA
G3PD
LAT
gB
pol
pol
Marker
F(5)
B(3)
B(1)
F(5)
B(3)
B(1)
F(5)B(3)B(1)
330 bp (pol)
191 bp (gB)
195 bp (LAT)

(a) Total RNA was extracted from three latently infected cell lines and was reverse-transcribed into cDNA. The same amounts from each cDNA sample were amplified for LAT, gB and pol, and the products were subjected to electrophoresis on a 2% agarose gel. G3PD was used as control and the presence of virus genome in DNA from the cell lines was assessed simultaneously. (b) cDNA and RNA samples without reverse transcription, amplified for G3PD, are shown to exclude the possibility that detected signals were due to contaminated DNA.

Lines revealed that CD8 antigen expression was down-regulated in all three cell lines, as shown in Fig. 5, whereas such membrane antigens as MHC class I, CD43 and CD45 remained unaffected; expression of HTLV-I p19 antigen also remained intact in these cell lines (data not shown). The level of CD8 expression showed little change during prolonged cultures of these cells, being highly suppressed in B(1) and B(3), while less so in F(5). Since the CD8 molecule plays an essential role as a co-receptor in antigen recognition, down-regulated expression of this molecule would directly impair the function of cytotoxic T lymphocytes and would be advantageous to the virus.

Encephalitogenic potential of infected lymphocytes

The encephalitogenic potential of infected cells was examined by injecting them i.v. into syngeneic B/Jas rabbits. B761.3, freshly isolated PBL and PHA-stimulated PBL, after being infected in vitro, were injected into a group of three rabbits, respectively. All animals were kept under observation for 1 month. The results are shown in Table 2. One to two animals in each group developed meningoencephalitic symptoms and virus was isolated from brain lesions of diseased animals. Although the frequency of disease incidence in rabbits given infected cells was lower and the onset of symptoms a little later than in rabbits given cell-free virus (Seto et al., 1995), these results suggested that infected lymphocytes transported virus to the brain. This possibility was examined in rabbits 3 days post-injection of infected B761.3 cells by PCR-amplifying the HTLV-I cellular junction sequence of B761.3. As shown in Fig. 6, the junction sequence was detected in PBL of all, and in the brain of one, of the three animals. Thus, there were aetiological implications of the involvement of infected lymphocytes in the development of herpetic encephalitis of B/Jas rabbits.

Discussion

HSV-1 has been shown to replicate in vitro in B as well as T lymphocytes (Kirchner et al., 1977; Westmoreland, 1978; Rinaldo et al., 1978). Virus replication occurs, however, not in
resting lymphocytes but in proliferating cells such as mitogen-stimulated and transformed cells. Thus, infectious viruses were rarely isolated from PBL, which were usually in the resting state: only a few cases of HSV-1 viraemia have been reported (Craig & Nahmias, 1973; Naraqui et al., 1976). In a recent study, HSV-1 DNA sequences were detected in blood and bone marrow cells, suggesting that these cells were latently infected (Cantin et al., 1994).

Two pathogenic pathways, haematogenous and neurogenic, have been postulated for HSV-1 dissemination through the body, and it is generally agreed that HSV-1 encephalitis results from neurogenic rather than haematogenous virus spread. HSV-2 spreads, on the other hand, via blood or presumably through infected lymphocytes to the meninges without affecting the brain. Development of meningoencephalitis following i.v. injection of infected lymphocytes, as observed in the present experiment, indicates a potential role for such cells in the pathogenesis of herpetic meningoencephalitis. Infected lymphocytes circulating in peripheral blood could have settled on the meninges and/or in the brain. The latent period before the appearance of meningoencephalitic symptoms was much the same for cell-free and lymphocyte-associated viruses, suggesting the direct infection of brain tissues by these agents. Alternatively, the virus may have infected peripheral nerves and been transported axonally up to the brain.

The KOS strain is usually categorized as non-neuropathogenic unless injected intracranially (Dix et al., 1983), but it could induce meningoencephalitis in B/5as rabbits when injected i.v. HSV-1 seems to be successfully transported to the brain/meninges in these particular rabbits, and lymphocytes may play a role in this transportation. In this regard, it seems pertinent to note that in viraemic cases of patients with herpetic meningitis, viraemia or lymphocyte-associated virus could be either the cause or the result of the meningitis (Craig & Nahmias, 1973). The present study experimentally showed that lymphocyte-associated HSV-1 could be the cause of herpetic meningoencephalitis.

The present experiment also allowed for the establishment of latently infected T cell lines. These cell lines did not produce infectious viruses, although they carried and partially expressed viral genes. A stable, persistent HSV-1 infection was reported for the human T lymphoblastoid line, CEM, which was established from an acute lymphoblastic leukaemia patient (Hammer et al., 1981). Virus expression in this cell line could be suppressed by neutralizing antisera and reactivated with PHA. Similar activation did not occur in our cell lines, suggesting that the states of HSV latency are not necessarily the same in all T cell lines. It is of interest, however, that double positive immature T cells of both human and rabbit origin could be latently infected with HSV-1. Cellular factors in these cells may play a role in latency by controlling the expression of virus regulatory proteins.

Infectious centre assays showed that the B(1) cell line contained a small proportion of producer virus, indicating that latency in B(1) was a type of low level infection, as observed frequently in cell culture. The same assay of B(3) and F(5) showed, however, that no producer virus was found in these cell lines. Repeated treatments with antibody plus complement should have totally eliminated producer virus, but the effects of these treatments do not seem to be confined to complement-dependent lysis of producer virus. Antibody and complement may have reacted with infected cells via viral antigens which were expressed (although undetectable by FACS analysis) on the membrane, and made these cells resistant to the hazardous effects of viral gene expression. The signal for this stimulation is presumably transmitted via viral glycoproteins not defined in the present experiment.

A classical study on the latent herpetic infection of neurons demonstrated that IgG antibody inhibited intraneuronal virus DNA and antigen synthesis, thereby restricting the appearance of infectious viruses (Stevens & Cook, 1974). A hypothesis was proposed accordingly that double binding of viral antigens by specific antibodies suppresses viral gene expression, thereby accounting for the necessity of IgG antibody in the maintenance of latency and giving the viral Fc receptor biological significance (Lehner et al., 1975). The possible roles of antiviral antibodies in latency have been discussed, and further studies will be needed to determine their validity (Rawls, 1985). The present experiment using culture cells showed that latency in two transformed lymphocyte cell lines could be maintained in the presence of fresh normal rabbit serum, the third cell line required antibody in addition. In these cell lines, three examined viral genes were variably expressed. Further analysis of viral gene expression in these cell lines is underway.

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References


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