Infection of Cultured Murine Brain Cells by Semliki Forest Virus: Effects of Interferon-αβ on Viral Replication, Viral Antigen Display, Major Histocompatibility Complex Antigen Display and Lysis by Cytotoxic T Lymphocytes

By A. MORRIS,* P. T. TOMKINS, D. J. MAUDSLEY AND M. BLACKMAN†

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.

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SUMMARY

Primary brain cell cultures prepared from newborn mice were infected with Semliki Forest virus (SFV). The effects of interferon (IFN-αβ) treatment on SFV replication, SFV and major histocompatibility complex (MHC) class I antigen expression, and susceptibility to lysis by SFV-specific cytotoxic T lymphocytes (CTL) were determined. The IFN-αβ treatment prevented replication of SFV as determined by incorporation of [3H]uridine into SFV RNA and very markedly reduced the expression of SFV antigens on the cell surface as determined by lysis with antibody and complement or indirect immunofluorescence. However, IFN-αβ increased expression of MHC class I antigens, measured by indirect immunofluorescence and as assessed indirectly by susceptibility to killing by alloreactive T cell lines. SFV infection had no effect on MHC class I expression in either IFN-αβ-treated or -untreated cells. The infected IFN-αβ-untreated brain cells were susceptible to killing by the CTL at effector/target ratios in the range 3 to 30. The killing was MHC antigen-restricted, and uninfected cells were not killed. A target cell (YAC) highly susceptible to natural killer cell cytotoxicity was not killed by the CTL. IFN-αβ treatment prior to SFV infection resulted in an augmentation of lysis by the CTL, indicating that even where SFV antigen expression is reduced, in the context of enhanced MHC class I expression brain cells remain susceptible to CTL killing.

INTRODUCTION

Infection of mice with virulent strains of Semliki Forest virus (SFV) which replicates to very high titres in the central nervous system (CNS) results in a severe encephalitis and death of the mice (Atkins et al., 1985). The mechanism of the encephalitis is unclear; it may be due either to direct cytopathic effects of the virus replication in brain cells (Gates et al., 1985) or to immune-mediated damage to infected cells. Evidence from reconstitution of SFV-infected nude mice with normal spleen cells (Fazakerley et al., 1983) and adoptive transfer of SFV-sensitized spleen cells (Berger; 1980) suggests a contribution of cell-mediated immunity to the pathological process.

There are several ways in which cell-mediated immunity might result in encephalitis. One of the most obvious is that SFV-specific cytotoxic T-lymphocytes (CTL) infiltrating into the infected brain could lyse infected brain cells. However, for CTL lysis to occur, the target cell must bear not only ‘foreign’ (in this case viral) antigen but also self major histocompatibility complex (MHC) class I antigens (Zinkernagel & Doherty, 1974, 1979). Brain cells normally express very little MHC class I antigens (Vitetta & Capra, 1978) and hence should be relatively insusceptible to CTL lysis. However, during SFV infection large amounts of interferon (IFN-αβ)
may be present in the brain (A. G. Morris & N. J. Dimmock, unpublished observations); IFN-\(\alpha/\beta\) is known to stimulate MHC antigen expression in a range of cell types, including brain cells (Wong et al., 1983). Therefore an obvious possibility is that IFN-\(\alpha/\beta\) produced during infection of mice by SFV stimulates MHC antigen expression in infected brain cells, thus making them more susceptible to lysis by CTL. We have previously shown that IFN-\(\alpha/\beta\) treated SFV-infected fibroblasts or lymphoblastoid cells (which normally express MHC antigens) remain susceptible to CTL despite complete inhibition of SFV replication in these cells (Blackman & Morris, 1985).

We have now extended these observations to primary brain cell cultures derived from newborn mice in order to study whether this occurs in SFV's major target cells. We find that IFN-\(\alpha/\beta\) treatment of these cells induces raised expression of MHC class I antigen; SFV replication is then blocked and the display of SFV antigens markedly reduced; however, CTL lysis is augmented.

METHODS

Viruses and cells. The avirulent strain of SFV (A774; Bradish et al., 1971) was used for immunization of mice and the virulent L10 strain for in vitro infection of target cells. Stocks of these viruses were prepared and titrated as previously described (Blackman & Morris, 1984).

L299 and C3H10T\(^4\) (Reznikoff et al., 1973) fibroblasts were cultured in the Glasgow modification of Eagle's MEM (GMEM; Gibco) supplemented with 10\% newborn calf serum (Gibco). Lymphoblastoid cells EL4 (H-2\(^b\)), RDM4 (H-2\(^k\)) and YAC (a natural killer cell target) were cultured in RPMI 1640 medium (Gibco) supplemented with 10\% foetal calf serum (Gibco).

Primary brain cell cultures were prepared from 1- or 2-day-old C3H-He or DBA mice (obtained from Olac, Bicester, U.K.) which are H-2\(^k\) and H-2\(^d\) respectively. Brains were removed from the mice, meninges separated by rolling on sterile filter paper and the tissues were disaggregated mechanically. The tissues were then reduced to single-cell suspensions by trypsinization. Sometimes deoxyribonuclease (Sigma) was used (20 \(\mu\)g/ml for 30 min at 37 \(^\circ\)C) to reduce viscosity due to released DNA. The resulting cell suspension was pelleted at low speed, washed [51Cr\(\mathrm{SO}_4\) from Amersham] at 20 gCi/ml overnight and the next morning mock infected or infected with SFV and then repelleted and resuspended in Dulbecco's modification of MEM (Gibco) supplemented with 10\% foetal calf serum. They were then planted in 5 cm Petri dishes (Nunclon, Roskilde, Denmark) at 2 \times 10^6 cells per dish.

Cells grew rapidly initially and were generally nearly confluent at 5 to 7 days after seeding, at which time they were used for experiments. Indirect immunofluorescence microscopy of permeabilized cells using anti-fibronectin (Calbiochem; from Cambridge Bioscience, U.K.) or anti-glial fibrillary acidic protein (GFAP) (Dako, High Wycombe, U.K.) showed there were few if any fibroblasts present and that at least the majority of the cells were GFAP+ astrocytes. This method of preparation of brain cells is essentially that of Bruce et al. (1984).

Cytotoxic T cells and cytotoxicity assays. SFV-specific and alloreactive (anti-H-2\(^k\)) CTL were prepared as previously described (Blackman & Morris, 1985). Brain cell cultures were treated with 1000 (reference research) units/ml of mouse IFN-\(\alpha/\beta\) (Lee Biomolecular, sp. act. 4 \times 10^7 units/ml, from Stratche Scientific, London, U.K.) for 2 days prior to cytotoxicity assays. Control cultures were left untreated. The cultures were treated with \(5\%^{51}\mathrm{Cr}\) from Amersham at 20 \(\mu\)Ci/ml overnight and the next morning mock infected or infected with SFV (m.o.i. 30, 300 or 1000 in different experiments). Cells were harvested by treatment with EDTA [0-02\% in phosphate-buffered saline (PBS)] for 15 min at 37 \(^\circ\)C, counted and distributed into the wells of 96-well microtitre plates at about 4 h post-infection to give effector : target (E : T) ratios ranging from 3:1 to 30:1. \(51\mathrm{Cr}\) release was determined after a further 5 h and specific release was calculated by the formula

\[
\text{lysis} = 100 \times \frac{(\text{release in wells at about 4 h post-infection to give effector : target (E : T) ratios ranging from 3:1 to 30:1.})}{(\text{total release - spontaneous release})},
\]

where total release was that in the presence of 0-5 m-hydrochloric acid.

In some experiments cytotoxicity against the lymphoblastoid cell targets EL4, RDM4 (infected or uninfected) or YAC (uninfected) was determined (depending on the numbers of effector cells available) to monitor cytotoxicity observed against the brain cell targets.

Alloreactive CTL killing was determined in essentially the same way but omitting the SFV infection.

Quantification of surface SFV and MHC class I antigens by specific antibody. SFV and MHC class I antigen expression on the surface of infected cells was determined in parallel to the cytotoxicity assays. SFV antigens were measured either by \(51\mathrm{Cr}\) release in the presence of SFV-specific antibodies and complement (Pel-Freez, from North-East Biomedical, Uxbridge, U.K.), or by indirect immunofluorescence, quantified by flow cytometry. MHC class I antigen expression was also measured by indirect immunofluorescence using antibodies to H-2\(^k\). The antibodies used were as follows. (i) A hyperimmune rabbit antiserum against SFV prepared by inoculation of purified SFV (Blackman & Morris, 1985). This antiserum showed no reactivity with uninfected brain cells or lymphoblastoid cells but some reactivity with uninfected fibroblasts. (ii) Monoclonal anti-H-2\(^k\) from hybridoma clone 11.4.1 (ATCC, TIB95). (iii) Monoclonal anti-H-2\(^D\alpha\) from clone 15-5-55 (ATCC, HB24) generously
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Fig. 1. Effect of IFN-αβ on incorporation of [3H]uridine into SFV RNA by L929 cells (○), C3H10T½ fibroblasts (▲) and cultured brain cells (■). Incorporation is expressed as a percentage of that by infected cells in the absence of IFN-αβ, with the background of residual cellular incorporation (i.e. by uninfected cells; 10% or less that of infected cells) subtracted.

provided as purified antibody by E. Culbert (ICI, Macclesfield, U.K.). (iv) FITC-conjugated sheep anti-rabbit Ig (Wellcome Diagnostics, Beckenham, U.K.). (v) FITG-conjugated goat anti-mouse Ig (Nordic Immunological Laboratories, Maidenhead, U.K.). These antibodies were all titrated and used at saturating concentrations. Flow cytometry was carried out using the Becton-Dickinson FACS 440 at Birmingham University, Birmingham, U.K. Cells were harvested and fixed with 3% paraformaldehyde in PBS prior to staining and stored at 4 °C in the dark after staining.

IFN-αβ sensitivity of astrocytes. To determine the relative sensitivity to IFN-αβ of SFV replication in C3H astrocytes these were distributed in 96-well microtitre plates at 4 × 10⁴ cells per well. Two C3H fibroblast lines (L929 and C3H10T½) both of which are highly sensitive to IFN-αβ were distributed in parallel. Next day the cultures were treated with a laboratory standard IFN-αβ preparation, 0.1 to 1000 units/ml in tenfold steps. The next day the cells were challenged with SFV (m.o.i. about 300) and labelled from 3 to 5 h post-infection with [3H]uridine (Amersham) in the presence of actinomycin D (Sigma). Under these conditions [3H]uridine is incorporated almost exclusively into SFV progeny RNA since cellular DNA-dependent RNA synthesis is inhibited. TCA-insoluble radioactivity was measured and plotted against IFN-αβ concentration (Atkins et al., 1974).

RESULTS

Relative sensitivity of C3H brain cells to IFN-αβ

Fig. 1 shows the incorporation of [3H]uridine into IFN-αβ treated, SFV-infected brain cells, L929 cells and C3H10T½ cells. The data show that all three cell types had about equal sensitivity to IFN-αβ, with about 50% inhibition of virus RNA replication at 1 unit/ml IFN-αβ and no RNA replication above background levels at 1000 units/ml. Hence 1000 units/ml was used for subsequent experiments.

Effect of IFN-αβ treatment on SFV antigen expression in brain cells measured by indirect immunofluorescence

Infection of brain cells with SFV resulted in a marked increase in reactivity with the anti-SFV serum (Fig. 2a and b) with a shift in the mean fluorescence intensity channel (MN) from 30 to 160 and a rise from 4 to 26% in the numbers of cells showing in the maximum fluorescence intensity channel (CH; maximum intensity is channel 255). Pretreatment of the cells with IFN-αβ however, very markedly reduced SFV antigen display (Fig. 2c) but did not abolish it; a small number of cells were still intensely stained (4% showing in maximum intensity channel) and the mean intensity was clearly elevated over levels seen in the uninfected cells.
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Fig. 2. SFV antigen expression by infected brain cells. SFV antigens were quantified by flow cytometry. Panels (a), (b) and (c) show the fluorescence intensity distribution for untreated, uninfected cells; untreated, infected cells; and IFN-αβ treated, SFV-infected cells respectively. In each case approximately 10⁴ cells were processed. MN, mean fluorescence of cells; CV, coefficient of variation; CH, fluorescence channel with largest number of cells in it (i.e. peak fluorescence); HI, number of cells in CH and as a percentage of total cells processed. Fluorescence intensity units are arbitrary, and the scale is linear from channel 0 to 255.

Effect of IFN-αβ treatment and SFV infection on expression of MHC class I antigens measured by indirect fluorescence and susceptibility to alloreactive CTL killing

Analysis by flow cytometry showed that the untreated, uninfected brain cells displayed undetectable levels of H-2K<k> since the fluorescence intensity of anti-H-2K<k>-stained cells was not significantly elevated above that of the same cells stained by an irrelevant antibody of the same class (data not shown). However, as others have found, H-2K<k> levels were markedly increased by IFN-αβ treatment of these cells (Fig. 3 a and b) with a shift in the mean fluorescence intensity channel from 42 to 85. SFV infection itself did not induce H-2K<k> expression in the cultured brain cells (Fig. 3 c) nor did SFV infection have any effect on the elevated levels of H-2K<k> expression in IFN-αβ treated cells (data not shown). Levels of H-2D<k> were similarly determined in brain cells treated with IFN-αβ. As with H-2K<k>, untreated brain cells showed no detectable HT2D<k> expression since again background fluorescence was essentially the same as for brain cells stained with an irrelevant antibody. However, brain cells treated with IFN-αβ showed strong expression of H-2D<k>, with mean fluorescence rising from channel 54 to channel 89 (Fig. 4). Hence expression of both major MHC class I antigens is increased in these brain cells by IFN-αβ treatment.

An indirect measure of H-2 expression by cells is their susceptibility to killing by alloreactive CTL, since these recognize only MHC class I determinants. Accordingly the effect of IFN-αβ
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Fig. 3. H-2K antigen expression by IFN-αβ-treated brain cells. MHC class I antigen (H-2Kk) was quantified by flow cytometry. Panels (a), (b) and (c) show the fluorescence intensity distribution for untreated, IFN-αβ-treated and SFV-infected cells respectively. In each case approximately 10⁶ cells were processed. Abbreviations as for Fig. 2.

Fig. 4. H-2D expression by IFN-αβ-treated brain cells. MHC class I antigen (H-2Dk) was quantified by flow cytometry. Panels (a) and (b) show the fluorescence intensity distribution for untreated and IFN-αβ cells respectively. In each case more than 14000 cells were processed. Abbreviations as for Fig. 2; S, number of cells sampled and as a proportion of the total.
Effect of IFN-αβ on lysis of infected brain cells. (a) Lysis by CTL of infected C3H (▲) or DBA (●) brain cells, untreated (—) or IFN-αβ-treated (—-), and uninfected non-IFN-treated YAC cells (■). (b) and (c) Killing of C3H and DBA brain cells respectively by anti-SFV antibody and complement; without (--) and with (+) IFN-αβ.

Table 1. Lysis of brain cells by alloreactive CTL

<table>
<thead>
<tr>
<th>Alloreactive CTL line</th>
<th>Without IFN</th>
<th>With IFN-αβ</th>
</tr>
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<tbody>
<tr>
<td>1 (2nd passage)</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>1 (3rd passage)</td>
<td>15</td>
<td>23</td>
</tr>
<tr>
<td>2 (3rd passage)</td>
<td>25</td>
<td>63</td>
</tr>
<tr>
<td>3 (3rd passage)</td>
<td>1</td>
<td>18</td>
</tr>
</tbody>
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* Brain cells were treated with IFN-αβ and subjected to lysis by alloreactive CTL as described in Methods. Data are quoted for an E:T ratio of 10:1.

Effect of IFN-αβ treatment on cytolysis of SFV-infected cultured brain cells by SFV-specific CTL or SFV-specific antibody and complement

In a series of experiments carried out with SFV-specific CTL prepared as above it was regularly found that infected syngeneic brain cells were killed efficiently irrespective of whether they were IFN-αβ-treated. In most of these experiments uninfected targets were not killed and nor were allogeneic targets (infected or uninfected), hence the killing by these effectors was specific and H-2-restricted as we have previously found with lymphoblastoid targets (Blackman & Morris, 1985). Occasionally the killing was unrestricted, and uninfected cells were also killed; in such experiments YAC cells were also killed, implying the presence of natural killer (NK) cells, and results of these experiments were disregarded. Killing of infected lymphoblastoid targets RDM4 and EL4 was parallel to killing of brain cell targets and quantitatively similar levels of lysis were obtained. In the experiment shown in Fig. 5 killing of infected C3H brain cells with or without IFN-αβ by CTL and antibody to SFV plus complement is shown. The IFN-αβ treatment resulted in somewhat increased CTL killing at all E:T ratios but abolished antibody and complement killing. This augmentation of CTL killing by IFN-αβ treatment of infected targets occurred in eight out of ten experiments; in the other two the killing was undiminished. The experiment shown in Fig. 5 also demonstrated that the CTL killing was MHC-restricted since infected DBA targets (whether or not IFN-αβ-treated) were not lysed whereas these were lysed by anti-SFV antibody and complement. YAC cells were not lysed, showing that there was no significant NK cell activity in the effector population. Therefore, brain cells treated with IFN-αβ and infected with SFV were killed by SFV-specific CTL as well.
as or better than non-IFN-αβ-treated brain cells, despite a marked diminution of SFV replication and antigen expression.

**DISCUSSION**

The importance of MHC antigen expression in immune recognition by T cells of their target cells is now well recognized. Cells that express no MHC class I antigens are not lysed by CTL (Zinkernagel & Doherty, 1979). Equally, 'foreign' antigens to which the T cell is sensitized must be present for recognition to occur; hence the T cell recognizes the foreign and self MHC antigen together. Since IFN-αβ regulates the expression of both viral antigens and MHC antigens, in opposite directions, the effects on CTL lysis of IFN-αβ treatment of target cells infected with a virus are likely to be complex. In previous experiments with fibroblasts and lymphoblastoid cells we have found that IFN-αβ treatment usually reduces but does not abolish CTL lysis of SFV-infected cells; others have found in different systems that IFN-αβ treatment can augment lysis (Bukowski & Walsh, 1985; Flyer et al., 1985). We have extended these experiments to brain cells because these cells normally express very low levels of MHC and so the effects of IFN-αβ treatment on CTL lysis may be more clear-cut. The study of T cell responses to brain cells is in itself of interest because of the possible role of T cell-mediated immunity in virus encephalitis and encephalopathies such as multiple sclerosis and subacute sclerosing panencephalitis.

We have found that cultured murine brain cells (predominantly GFAP+ astrocytes) infected with SFV are susceptible to lysis by CTL. The fact that this lysis is MHC-restricted very obviously implies that there is sufficient MHC expression for T cell recognition to occur, although this could not be detected by indirect immunofluorescence. It was possible that infection with SFV induced MHC expression during the cytotoxicity assay thus allowing CTL recognition. Considering the short length of time involved this does seem unlikely; however, retroviruses, at least, can induce MHC antigens (Flyer et al., 1985) and so can coronavirus (Massa et al., 1986) so this also was a formal possibility in the case of SFV infection. The flow cytometric data show that H-2Kk was not induced by SFV infection, but that does not exclude the possibility that some other restriction element may be involved. However, the simplest explanation for the data is that MHC antigens are present in amounts sufficient for CTL recognition but insufficient for detection by antibody. The experiments in which we have found alloreactive CTL to lyse brain cells tend to confirm the presence of MHC antigens.

The IFN-αβ treatment of the brain cells reduced SFV replication and SFV antigen expression to a very low level but did not completely abolish SFV antigen expression. H-2 expression on the other hand was increased as was shown by increased staining with monoclonal antibodies reactive with H-2Kk and H-2Dk and by increased susceptibility to lysis by alloreactive CTL. The net result was that although lysis by antibody and complement was essentially abolished, CTL lysis was somewhat augmented. This could well play an important role in T cell-mediated mechanisms of SFV-induced encephalitis in mice.

Since our primary brain cell cultures are mixed, although predominantly GFAP+ astrocytes, we cannot at present determine in which cell type SFV replication mainly occurs nor which type is the major target for CTL. However, since up to 80% of cells displayed SFV antigens and up to 80% lysis by CTL occurred, then at least some of the astrocytes must express virus antigens and are killed. Whether other cells (e.g. oligodendrocytes), making up a minor proportion of the cultures, are also killed remains to be determined.

These results have a number of significant implications for resistance to virus infections, especially for infections of the CNS. Provided IFN is present to increase MHC expression, CTL (or other T cell) responses occur even though virus replication is limited and virus components may be present at very low levels. Once such a response is triggered, it could be self-perpetuating; in response to virus-infected target cells T cells produce IFN-γ (Morris et al. 1982; Blackman & Morris, 1984) which is particularly effective at augmenting MHC expression and so despite its effect in limiting virus replication the T cell response could continue. Preliminary results employing recombinant murine IFN-γ prepared in this laboratory from genetically engineered Chinese hamster ovary cells indicate that IFN-γ also augments CTL lysis of IFN-treated, SFV-infected brain cells, in a way very similar to that described above for natural IFN-
α/β. The production of IFN-γ by T cells responding to virus-infected brain cells could precipitate autoimmune responses to normal CNS components. Thus, it has been shown that IFN-γ augmenting H2-Ia expression by astrocytes enables these cells to present myelin basic protein (MBP) to T cells (Fierz et al., 1985). T cells thus sensitized to MBP could mount an autoimmune attack on the CNS (Zamvil et al., 1985). Hence, a CNS virus infection could trigger in this way diseases such as multiple sclerosis. In such a case one might expect IFN treatment of multiple sclerosis to exacerbate the disease; however, at least IFN-α/β does not (Jacobs et al., 1982).

Our results overall emphasize the importance of histocompatibility antigens in immune recognition of virus-infected cells and also suggest an important role for IFN-α/βs and virus infections in autoimmune diseases of the CNS.

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REFERENCES


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