Theiler's murine encephalomyelitis virus-binding activity on neural and non-neural cell lines and tissues

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Three categories of cell lines are described with respect to their activity in binding Theiler's murine encephalomyelitis virus (TMEV). High, medium and low densities of viral receptors can be detected on cell lines from different species and origins by using an immunological binding assay. Nevertheless, TMEV acts as a fastidious virus that only infects a few cell types productively. No correlation between virion binding and degree of permissiveness to infection could be detected. The presence of viral receptors in both susceptible and resistant strains of mice seemed to have a widespread tissue distribution, the thymus being an exception. When primary cerebral cultures, enriched in neurons, astrocytes or oligodendrocytes, were checked in the immunological assay, a higher density of viral receptors was detected in the neuronal population. The number of virus-binding sites in the BHK-21 cell line is reported here to be $5 \times 10^3$ per cell; approximately $15 \times 10^3$ and $2.5 \times 10^3$ are the estimates of binding sites per cultured neuron and macroglial cell, respectively.

Introduction

The first step for successful replication of an animal virus includes attachment to cell receptors on the plasma membrane (Bukrinskaya, 1982; Dimmock, 1982; Kohn, 1979). If all the stages of productive replication are accomplished, progeny particles are produced. The receptors for two picornaviruses, poliovirus (Mendelsohn et al., 1989) and the major human rhinovirus (Greve et al., 1989; Stauton et al., 1989), were recently identified as members of the immunoglobulin superfAMILY, a set of a large number of cell surface components of mammalian cells that participate in cellular recognition and adhesion. Some controversy has arisen in the past about the neural cells that are productively infected by Theiler's murine encephalomyelitis virus (TMEV), a picornavirus that induces in susceptible mice a disease similar to human multiple sclerosis (Dal Canto & Lipton, 1982). Immunohistochemical results from intracerebrally inoculated SJL mice indicate that in the chronic phase of the disease almost all the central nervous system (CNS) cell populations are affected (Dal Canto & Lipton, 1982). On the other hand, intraperitoneal injection leads to a widespread infection of most organs examined (Rodriguez et al., 1983).

Therefore, still unresolved is the question of whether TMEV attaches only to some specific populations of cells or whether the receptors for such a virus are widely distributed. One approach for resolving this issue would be to test cell lines and tissues in vitro for the presence of TMEV-binding activity.

We have performed such a study by using a new sensitive method developed by ourselves (Rubio & Cuesta, 1988), in which the binding of viral particles to cell receptors was detected by using anti-viral antibodies and $^{125}$I-labelled Protein A. The infectious centre assay allowed us to determine the correlation between the presence or absence of cell receptors and the real capacity to induce a productive infection.

Methods

Cell lines. The cell lines used are listed in Table 1. Most of them derive from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and gentamicin (Flow Laboratories). EL4 and CTLL lymphocytic cell lines were cultured in RPMI 1640 medium containing 10% FCS. The CTLL medium also contained 20% mouse spleen medium conditioned with 5 μg/ml concanavalin A, as a source of interleukin 2 (Gearing et al., 1985).

Tissue cell suspensions. Different tissues were removed from newborn mice after cervical dislocation, carefully dissociated by passing through stainless steel screens in DMEM containing 0.1% bovine serum albumin (BSA) (Sigma), washed twice by centrifugation and counted in trypan blue solution. Only highly viable cell suspensions were used in this study.
Table 1. TMEV receptors on different cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Species</th>
<th>Maximum c.p.m. bound (± S.E.M.)*</th>
<th>n†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21‡</td>
<td>Fibroblast</td>
<td>Hamster</td>
<td>18000 ± 1500</td>
<td>10</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervical carcinoma</td>
<td>Human</td>
<td>17400 ± 1100</td>
<td>4</td>
</tr>
<tr>
<td>C-6</td>
<td>Glioma</td>
<td>Rat</td>
<td>17000 ± 950</td>
<td>2</td>
</tr>
<tr>
<td>Neuro-2A</td>
<td>Neuroblastoma</td>
<td>Mouse</td>
<td>16500 ± 900</td>
<td>2</td>
</tr>
<tr>
<td>Vero</td>
<td>Fibroblast</td>
<td>Monkey</td>
<td>9500 ± 420</td>
<td>4</td>
</tr>
<tr>
<td>L-929</td>
<td>Fibroblast</td>
<td>Mouse</td>
<td>8400 ± 400</td>
<td>3</td>
</tr>
<tr>
<td>J774</td>
<td>Macrophage</td>
<td>Mouse</td>
<td>7000 ± 350</td>
<td>2</td>
</tr>
<tr>
<td>K-BALB</td>
<td>Fibrosarcoma</td>
<td>Mouse</td>
<td>6300 ± 320</td>
<td>2</td>
</tr>
<tr>
<td>Clone M3</td>
<td>Melanoma</td>
<td>Mouse</td>
<td>6200 ± 320</td>
<td>2</td>
</tr>
<tr>
<td>LL-2</td>
<td>Lung carcinoma</td>
<td>Mouse</td>
<td>5500 ± 280</td>
<td>2</td>
</tr>
<tr>
<td>CTLL§</td>
<td>T cytolytic</td>
<td>Mouse</td>
<td>3000 ± 2600</td>
<td>2</td>
</tr>
<tr>
<td>EL4-6.1§</td>
<td>Thymoma</td>
<td>Mouse</td>
<td>1450 ± 220</td>
<td>3</td>
</tr>
</tbody>
</table>

* Input was 100000 c.p.m.
† Number of determinations.
‡ All cell lines reached a density of around 700000 cells per Petri dish.
§ Background binding reached a mean of 1.5 to 2% of input (1500 to 2000 c.p.m.) for all experiments.

Virus and viral titration. A strain of TMEV isolated from a ferret mouse in 1975 in Belem (Brazil), called BeAn 8386, was passaged in BHK-21 cells.

Viral titration was performed by plaque assay on confluent monolayers of cells. Briefly, 10-fold dilutions of virus in DMEM with 0.1% BSA were adsorbed for 60 min. Following adsorption, the cells were washed and overlayed with medium containing 1% Noble agar (Difco Laboratories) and incubated at 33 °C. The plaques were fixed in 1% formaldehyde. After incubation with rabbit anti-glial fibrilar acidic protein antiserum (Dakopatts) and immunofluorescence staining of methanol-fixed cultures by using FITC-labelled goat anti-rabbit IgG (Miles Laboratories), the content of oligodendrocytes was greater than 95%, as determined by indirect immunofluorescence of living cultures by using guinea-pig anti-myelin basic protein serum (Rubio & Cuesta, 1989), and fluorescein isothiocyanate (FITC)-labelled goat anti-guinea-pig IgG (Sigma).

Astrocyte-enriched cultures. The astrocyte cultures were prepared by mechanical dissociation of the cerebral cortex from newborn SJL and BALB/c mice, and Wistar rats. Cell suspensions were filtered through 80 μm pore size mesh into DMEM containing 10% FCS (Manthorpe et al., 1979). After centrifugation, cells were filtered through a 20 μm mesh sieve, plated in tissue culture flasks (75 cm²) in an orbital shaker. The suspended cells were collected and filtered through a 33 μm mesh sieve in order to remove small clumps of astrocytes, centrifuged (5 min at 40 g) and plated in 35 mm Petri dishes.

The content of oligodendrocytes was greater than 95%, as determined by indirect immunofluorescence of living cultures by using guinea-pig anti-myelin basic protein serum (Rubio & Cuesta, 1989), and fluorescein isothiocyanate (FITC)-labelled goat anti-guinea-pig IgG (Sigma).

Results

Binding activity on cell lines

A total of 12 cell lines was checked for the presence or absence of receptors for TMEV. Table 1 shows that the cell lines tested can be grouped into three categories according to the density of receptors available on the cell surface. The first group displayed a high level of viral binding. BHK-21, the cell line that usually maintains TMEV replication in tissue culture and is used to passage mouse viruses from brain isolates, had full expression of receptors. HeLa cells (a human cervical carcinoma) and the two cell lines of neural origin, the neuroblastoma Neuro-2A and the astrocytoma C-6, display the same binding capacity.
A second group containing cell lines with a medium receptor density, around two to three times lower, included the monkey Vero fibroblast, the mouse fibroblast L-929 and the macrophage-like J774 cell lines. Three mouse tumours (a melanoma, a fibrosarcoma and a lung carcinoma) also show intermediate levels of receptors. Two cell lines of T lymphocytic origin, the EL4 thymoma and the T cytolytic clone CTLL, were found negative.

Tissue distribution of viral receptors

We have evaluated the presence of receptors for TMEV on a variety of non-neural tissues and whole brain for both susceptible (SJL) and resistant (BALB/c) strains of mice. Tissues were selected to represent brain populations, immunological tissues, visceral organs and muscles. Results of the binding assay are presented in Table 2. The thymus seems to be the only organ that did not show an appreciable number of virus receptors. Maximal density of receptors appears to be present in the brain, followed by liver, kidney, lung and spleen; a lower density is seen in muscle and the small intestine. All of these organs have a highly developed reticuloendothelial system and/or mucosa-associated lymphoid tissue, but the involvement of specific cells from either system was not investigated.

Therefore, making an exception of the thymus, a widespread distribution of receptors seems to be indicated. The lysis of erythrocytes by 0.8% ammonium chloride (10 min in ice) and further washing of the membranes by centrifugation, did not alter the binding capacity of the tissues, indicating that red cells do not contribute to the binding capacity of the different tissues. Previous results from other authors indicate that virus could be isolated from all major organs examined after intraperitoneal injection (Rodriguez et al., 1983). A higher binding capacity was consistently found in cells in suspension than in cells attached to plastic. This can be explained because the surface exposed for viral binding is greater on free cells in suspension.

BALB/c, a strain resistant to TMEV-induced disease, has the same density and tissue expression of receptors as SJL, suggesting that the multiple sclerosis-like disease does not appear to be related exclusively to the ability of TMEV virus to bind to cells.

**Table 2. TMEV receptors on different mouse tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Maximum c.p.m. bound ± S.E.M.</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJL strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain*</td>
<td>4150 ± 2600</td>
<td>4</td>
</tr>
<tr>
<td>Lung</td>
<td>21900 ± 1800</td>
<td>3</td>
</tr>
<tr>
<td>Kidney</td>
<td>20000 ± 1700</td>
<td>3</td>
</tr>
<tr>
<td>Liver</td>
<td>20850 ± 1700</td>
<td>3</td>
</tr>
<tr>
<td>Spleen</td>
<td>21570 ± 1820</td>
<td>3</td>
</tr>
<tr>
<td>Muscle</td>
<td>15200 ± 800</td>
<td>2</td>
</tr>
<tr>
<td>Small intestine</td>
<td>15000 ± 800</td>
<td>2</td>
</tr>
<tr>
<td>Thymus</td>
<td>2500 ± 350</td>
<td>3</td>
</tr>
<tr>
<td>BALB/c strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>43200 ± 2700</td>
<td>2</td>
</tr>
<tr>
<td>Lung</td>
<td>21000 ± 1800</td>
<td>2</td>
</tr>
<tr>
<td>Kidney</td>
<td>20000 ± 1750</td>
<td>3</td>
</tr>
<tr>
<td>Liver</td>
<td>23000 ± 1950</td>
<td>3</td>
</tr>
<tr>
<td>Spleen</td>
<td>14000 ± 750</td>
<td>2</td>
</tr>
<tr>
<td>Muscle</td>
<td>18000 ± 1600</td>
<td>3</td>
</tr>
<tr>
<td>Small intestine</td>
<td>12200 ± 700</td>
<td>2</td>
</tr>
<tr>
<td>Thymus</td>
<td>3000 ± 400</td>
<td>2</td>
</tr>
</tbody>
</table>

* Number of determinations.
† For all tissues 1 x 10⁶ cells per test tube were used.
‡ Background binding reached a mean of 2 to 2.5% of input (2000 to 2500 c.p.m.) for all experiments.

Virion binding on neural cell cultures

Immunohistochemical studies demonstrate in this system a state of virus persistence in CNS, in which discrete areas of virus-infected cells coexist among uninfected tissue (Dal Canto & Lipton, 1982).

We have studied the binding of TMEV by purified neurons, astrocytes and oligodendrocytes maintained in tissue culture. Neurons from the cortex of mouse and rat embryos showed the higher binding capacity of the three neural populations maintained in tissue culture (Fig. 1). The level of radioactivity bound per dish (15000 to 16000 c.p.m.) was similar to those bound by the high density receptor cell lines, including the mouse neuroblastoma Neuro-2A (Fig. 1 and Table 1). In all primary cultures, the cells reached semiconfluent densities, that roughly
correspond to 0.3 mg of total protein per 35 mm diameter dish for neurons and 1.2 mg for astrocytes, as determined by using the method of Lowry et al. (1951). Previous results from other authors also show neurons as primary targets of TMEV infection, as demonstrated by using immunohistochemical and in situ hybridization techniques (Lipton, 1975; Stroop et al., 1981; Zurbriggen & Fujinami, 1988).

Astrocyte-enriched cultures demonstrate a clear but low binding capacity for TMEV (Fig. 1). This was suggested by previous in vivo results (Dal Canto & Lipton, 1982), which demonstrated that astrocytes are infected only in a second phase of the disease induced by intracerebral inoculation of the virus and that neurons are the main target cell in TMEV infection.

Concerning the oligodendrocyte-enriched cultures, their binding capacity is the same as the astrocytes, in agreement with recent results from Rodriguez et al. (1988). To establish all these conclusions, care has been exercised to ensure that the cells from primary cultures were employed at approximately equal, uniform densities. Once more, no differences were shown between cells from susceptible (SJL) or resistant (BALB/c) strains of mice or rat.

Furthermore, we have studied virus-receptor interactions by means of the Scatchard plot, where the amounts of bound/free virus were plotted against that of the bound virus, as determined by c.p.m. of Protein A counted (Fig. 2). Analysis of the plot reveals that only one binding site with the same affinity occurs in the BHK-21 cell line and in the primary cultured neurons, astrocytes and oligodendrocytes. This is indicated by the production of straight lines with the same slopes. The intercept with the x-axis, giving the number of binding sites per milligram of protein, showed that the neurons have three times more receptors than BHK-21 and six times more than astrocytes and oligodendrocytes.

The observation that the BHK-21 cell line reached saturation at 10 p.f.u. per cell (Rubio & Cuesta, 1988) indicated that it contains around 5000 virus sites per cell, as 500 virion particles are needed in this system to obtain 1 p.f.u. According to the above calculations, the neurons have $15 \times 10^3$ receptors per cell, and astrocytes and oligodendrocytes have around $2-5 \times 10^3$ each.

![Fig. 1. TMEV-binding activity of neurons (○), astrocytes (■), or oligodendrocytes (▲) from rat or SJL and BALB/c mice. The amount of radioactivity bound was plotted as a function of the number of p.f.u. added per Petri dish. Results are the mean values of three experiments.](image1)

**Discussion**

There are at least two non-H-2 genes involved in the differential susceptibility of mouse strains to TMEV-induced disease (Melvold et al., 1989). These host genes may determine the presence and density of cell surface receptors, the RNA transcriptional machinery and the post-transcriptional processing of viral polyprotein precursors. In addition, it is known that neurovirulence depends on the ability of a particular virus to bind to and replicate in a specific cell population in the CNS and that
the two TMEV subgroups of viruses replicate in different populations of neurons (Lipton, 1975).

In this study we have specifically focused on the early interactions occurring between TMEV and plasma membranes of several cell lines and mouse tissues. The cell lines tested for virion attachment showed a gradient of receptor density, being maximum for BHK-21, HeLa cells and cell lines of neural origin (C-6 and Neuro-2A), and minimum in cell lines of T lymphocyte origin (CTLL and EL4). Other cells, of tumoral and epidermal origin, showed an intermediate activity.

Cellular resistance to viral infection may result from a deficiency of virus-specific receptors (Jondall & Klein, 1983; McLaren et al., 1959; Tardieu et al., 1986) or be due to post-transcription steps (Krontikis et al., 1973; Piraino, 1967; Steck & Rubin, 1966). In the present system, where a detectable presence of receptors on several cell lines did not lead to a productive infection, the second situation seemed to apply.

Concerning the tissue distribution, most of the cells from different organs have receptors, except those from the thymus (Table 2). A similar range of receptor expression in human tissues has been described for poliovirus (Mendelsohn et al., 1989). There is a good correlation between organs and the tissue origin of the cell lines used, as lymphoblastoid cell lines of T cell origin also lack receptors (Table 1). Although neurons and thymocytes share the Thy-1 antigen (Reif & Allen, 1964), this is not the case for the components on the surface of the cell that recognize the TMEV particle.

Intraperitoneal infection of experimental animals led to the isolation of virus from most organs (Rodriguez et al., 1983). TMEV also replicates in muscle after intramuscular injection (Rustigian & Pappenheimer, 1949) and, after infection by feeding, the virus reached high titres in the brain and spinal cord, but moderate amounts of virus were found in the visceral organs: the lung, gastrointestinal tract, liver and spleen (Liu et al., 1967). These titres declined during the ensuing days. This indicated the presence of receptors in such organs, although primary viral replication occurred only in the CNS and muscle.

Binding studies performed with purified populations of neural cells maintained in tissue culture demonstrated that neurons are the main target of the infective TMEV virions (Fig. 1 and Fig. 2). The number of virus-binding sites on neurons are calculated to be 15 x 10^3. Glial cells have around 2.5 x 10^3 specific receptors per cell, half the number of receptors on the surface of BHK-21 cells. The correlation between the binding results from primary cultures and established cell lines (Neuro-2A and C-6) demonstrated a real binding capacity of such specific cell populations and was not an effect due to the contamination by other cells of the enriched primary cultures.

In summary, a widespread distribution of receptors for TMEV was demonstrated. A higher number of binding sites on neurons compared with glial cells was found. There was no difference between strains of mice in connection with the presence of receptors, indicating that the multiple sclerosis-like disease induced by the BeAn 8386 strain of TMEV was not due to direct viral replication and cytopathic damage but to secondary mechanisms, probably of immune origin (Lipton & Dal Canto, 1976). We conclude that the two genes controlling the susceptibility of different strains of mice to the TMEV-induced demyelination do not encode cell surface viral receptors, and that the expression of the viral receptor may be necessary but is certainly not sufficient for either virus replication or pathogenesis.

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References


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