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Overexpression of basal c-*fos* and c-*jun* but not of *ras* oncogenes after Theiler's murine encephalomyelitis virus infection of glial cells

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Constitutive expression of the cellular proto-oncogenes c-*fos* and c-*jun*, and in a lesser extent *ras*, was demonstrated in the glioma cell line C-6 by flow cytometry analysis using specific mono and polyclonal antibodies. Basal expression of the products of the early response genes c-*fos* and c-*jun* were increased 66 and 50% when Theiler's murine encephalomyelitis virus (TMEV) infected these cells. No increase in *ras* transcription could be demonstrated after infection. This activation follows a kinetic reaching maximum values after 60 min and was proportional to the multiplicity of infection used. The described effect was completely abrogated by rabbit antibodies to TMEV but was not altered by normal rabbit serum. Furthermore, an intact infectious virion is needed to detect this effect. Fetal calf serum and lipopolysaccharide stimulation slightly increases c-*fos* and c-*jun* expression following a slower kinetics. Cytokine treatment (IL-1α, IL-6, IFN-γ and TNFα), did not induce oncogene overexpression. Therefore, this stimulation seems to be linked to the TMEV infectious process.

Keywords: c-*fos*; c-*jun*; *ras*; Theiler's virus; glial cells

Introduction

Induction or over-induction of c-*fos* and c-*jun* proto-oncogenes is part of the transient early response to stimuli that provoke cell proliferation and differentiation. c-*fos* has been reported as playing a role in differentiation of muscle cells (Trouche *et al*, 1993), macrophages (Collart *et al*, 1987) and megakaryocytes (Panterne *et al*, 1992) and also in programed cell death (apoptosis) (Smeyne *et al*, 1993). c-*jun* has been reported as being essential for normal mouse development (Hilberg *et al*, 1993).

In the central nervous system (CNS), the *fos/jun* complex is also involved in development processes. Basal expression of both transcription factors was located in rat hippocampus (Hughes *et al*, 1992) and cortex (González-Martin *et al*, 1992). Transient expression of c-*fos* was demonstrated during development of cortex (González-Martin *et al*, 1991) and both immediate early genes were induced by nerve transection (Herdegen *et al*, 1990), inflamation (Noguchi *et al*, 1992), and electrical stimulation (Fu and Beckstead, 1991). The induction of c-*fos* by neurotransmitters in cultured astrocytes has also been reported (Arenander *et al*, 1989).

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c-*fos* expression is induced in target cells by several cytokines, as interleukin-2 (IL-2) (Hatakeyama *et al*, 1992), IL-6 (Körholz *et al*, 1992), IL-1α (Kovacs *et al*, 1986) and TNFα (Lin and Vilcek, 1987). In the same way, TNFα (Brenner *et al*, 1989) and IL-1 (Muegge *et al*, 1989) induces *jun* expression that is also activated by adenovirus (Hagmeyer *et al*, 1993), visna virus (Shih *et al*, 1992) or the proteins of hepatitis B virus (Twu *et al*, 1993).

The *ras* superfamily regulates many aspects of differentiation and cell growth (Barbacid, 1987; Boguski and McCormick, 1993). In the CNS, prolonged *ras* activity is associated with neuronal differentiation (Qiu and Green, 1992) and is regulated by multiple intragenic elements (Jeffers and Pellicer, 1992).

Theiler's virus is a picornavirus which induces a demyelinating disease in susceptible mouse strains. This is currently used as an experimental model of multiple sclerosis (Lipton and Friedman, 1980; Dal Canto and Lipton, 1982; Roos and Wollmann, 1984). Furthermore, TMEV is a potent inducer of cytokines in astrocyte cultures (Rubio and Capa, 1993; Rubio and Sierra, 1993; Sierra and Rubio, 1993) and persistently infects glioma cells (Patick *et al*, 1990). In the present article, we have studied the effect of TMEV in the stimulation of the constitutive expression of c-*fos*, c-*jun* and *ras* oncogenes in the established cell line of glial origin (C-6).

Results

Staining controls

Because of the double localization of the proteins products studied, both nuclear (c-*fos* and c-*jun*) and cytoplasmic (*Ras*), positive and negative controls were chosen carefully. Accessibility of antibodies to the nuclear localization was demonstrated in our permeabilization conditions using an anti-histones monoclonal antibody. This antibody stains nuclei of cells of diverse origins, including mouse cells (Fujita *et al*, 1983) and provides an efficient positive control performed in all experiments (Figure 1B). To demonstrate positive cytoplasmic staining (in addition to a productive infection of C-6 glioma cells by TMEV), we stained cells infected 24 h before with a rabbit anti purified TMEV which contains antibodies against VP1 and VP2 virion structural proteins (Clatch *et al*, 1987). This positive cell staining (Figure 1A) demonstrates cytoplasmic replication of TMEV 24 h post-infection.

As a negative control, cells incubated with an irrelevant antibody (a monoclonal antibody against mouse Mac-1 antigen) were included in each experiment (Figure 1C). We further considered an experiment positive when fluorescence intensity was above the upper limit of the negative control. Therefore, we conclude that fixation with 3.7% formaldehide and permeabilization using 0.1% Triton X-100, retains antigenic integrity in nucleus and cytoplasm with no significant increase in non-specific background fluorescence neither autofluorescence. Optimal concentrations of both primary and secondary antibodies were determined experimentally.

Constitutive expression

Constitutive expression of c-*fos*, c-*jun* and *ras* oncogenes was demonstrated even in C-6 cells made quiescent by culture for 48 h in 0.25% FCS and thus depleted of growth factors. The more actively expressed is c-*fos*. We obtained stainings with a mean channel number of 120, followed by c-*jun* with an intensity of 60 (Figure 2A and B). The intensity of *ras* constitutive expression shows a mean fluorescent intensity of 9 (Figure 2C) which is nevertheless positive if it is compared with the negative controls (Figure 1C). Further data on overexpression results were represented in the figures with a cursor set to the right of the constitutive oncogene expression.

TMEV induces overexpression of c-fos *and* c-jun

An early increase of c-*fos* and c-*jun* as a response to TMEV infection was demonstrated by FACS analysis (Figure 3A and B). In the case of c-*fos*, a 66% increase in fluorescence intensity (constitutive expression =100%) was obtained when a moi of 20 was tested (from a channel number of 120 to 200). This increase was of 30% when using a moi of 5, and decreases to control values when the moi was 1 (data not shown). The effect described was saturable, as no further augmentation in fluorescence intensity resulted when moi greater than 20 were tested.

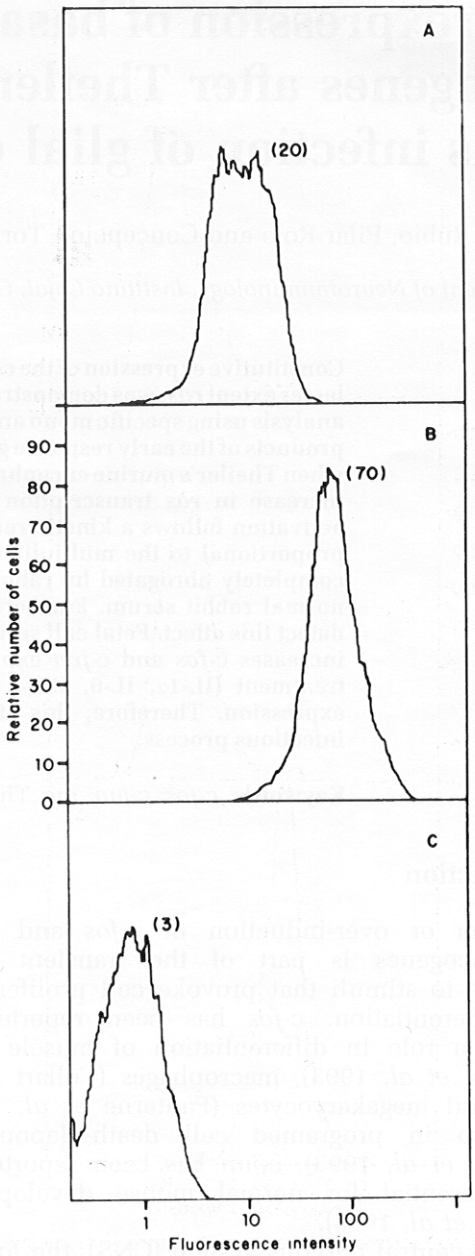

Figure 1 Positive and negative controls for our immunofluorescence analysis conditions. (A) Positive cytoplasmatic staining with rabbit anti-TMEV, 24 h after infection. (B) Positive nuclear staining of cells with monoclonal anti-histones antibody. (C) Negative staining control with an irrelevant antibody (anti Mac-1 monoclonal antibody). In each histogram, numbers in parentheses represent mean fluorescence intensity.

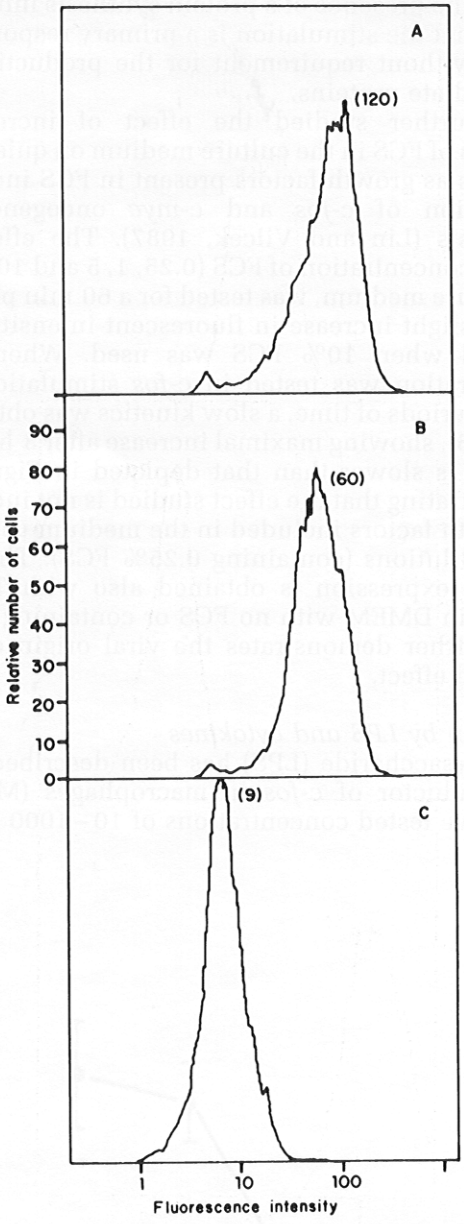

Figure 2 Constitutive expression of the studied oncogenes in C-6 quiescent cells (A) c-*fos*. (B) c-*jun*. (C) *ras*.

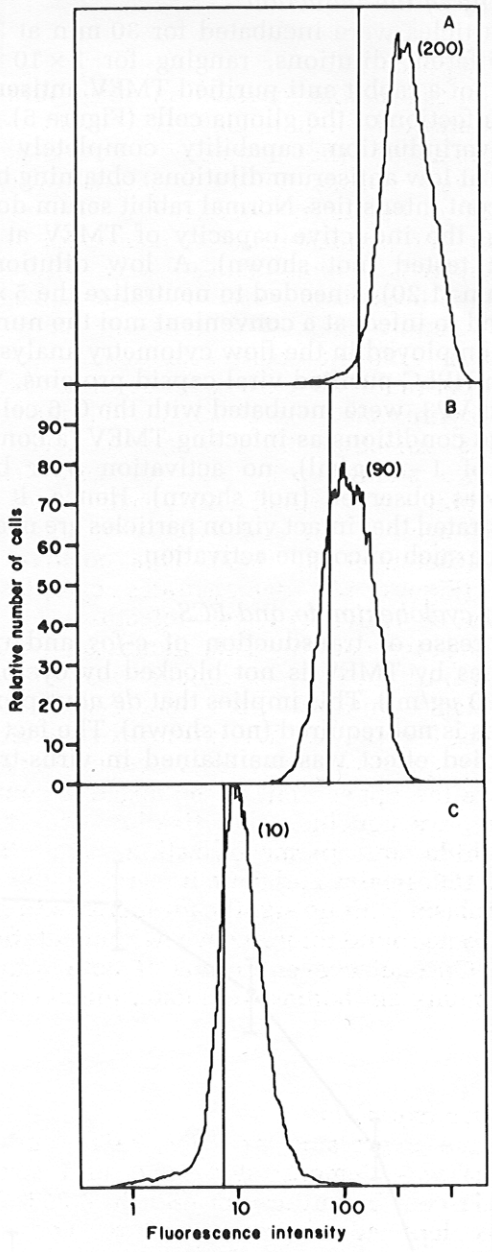

Figure 3 Overinduction on (A) c-*fos*, (B) c-*jun* and (C) *ras* by TMEV infection. The cursor is set to the right of the constitutive expression values depicted in Figure 2.

The maximum increase in fluorescence intensity values was of 50% in the case of c-*jun*. *ras* proto-oncogene expression is not activated over constitutive expression, despite the amount of virus used.

Time-course overinduction

To further assess the nature of the above effect, the time-course overexpression after viral infection, was measured at 37°C. Figure 4 shows changes in fluorescence intensity using a moi of 20. The constitutive fluorescent intensity was subtracted from the values obtained after several incubation periods. Virus samples were previously equilibrated at 37°C and allowed to bind to its receptor for a 15 min period (absorption). A clear increase can be detected 15–30 min after the absorption period is finished. An equilibrium is reached at 60 min, followed by a return to background levels by 6 h. As for Figure 3 overexpression of c-*fos* is more pronounced than that of c-*jun*. No increase of *ras* expression was detected, independently of the incubation time in the presence of TMEV.

Specificity of the induction

Viral particles were incubated for 30 min at 37°C with different dilutions, ranging for 2×10^{-1} to 2×10^{-4}, of a rabbit anti purified TMEV antiserum, before infection of the glioma cells (Figure 5). The viral overinduction capability completely disappears at low antiserum dilutions, obtaining basal fluorescent intensities. Normal rabbit serum do not decrease the inductive capacity of TMEV at any dilution tested (not shown). A low dilution of antiserum (1:20) is needed to neutralize the 5×10^5 PFU used to infect at a convenient moi the number of cells employed in the flow cytometry analysis.

When HPLC-purified viral capsid proteins, VP1, VP2 and VP3, were incubated with the C-6 cells in the same conditions as infecting TMEV (a concentration of 1–4 ng/ml), no activation over basal levels was observed (not shown). Hence, it was demonstrated that intact virion particles are needed to induce such oncogene activation.

Effect of cycloheximide and FCS

The increase of transduction of c-*fos* and c-*jun* oncogenes by TMEV is not blocked by cycloheximide (20 μg/ml). This implies that *de novo* protein synthesis is not required (not shown). The fact that the studied effect was maintained in virus-trated cells in the presence of a protein synthesis inhibitor shows that the stimulation is a primary response to TMEV without requirement for the production of intermediate proteins.

We further studied the effect of increasing amounts of FCS in the culture medium on quiescent C-6 cells as growth factors present in FCS induced stimulation of c-*fos* and c-*myc* oncogenes in fibroblasts (Lin and Vilcek, 1987). The effect of several concentration of FCS (0.25, 1, 5 and 10%) in the culture medium, was tested for a 60 min period. Only a slight increase in fluorescent intensity was detected when 10% FCS was used. When this concentration was tested in c-*fos* stimulation for longer periods of time, a slow kinetics was obtained (Figure 6), showing maximal increase after 3 h. This kinetics is slower than that depicted in Figure 4, demonstrating that the effect studied is not induced by growth factors included in the medium used for TMEV dilutions (containing 0.25% FCS). The fact that overexpression is obtained also with TMEV diluted in DMEM with no FCS or containing 0.1% BSA, further demonstrates the viral origin of the observed effect.

Induction by LPS and cytokines

Lipopolysaccharide (LPS) has been described as a rapid inductor of c-*fos* in macrophages (Müller, 1986). We tested concentrations of 10–1000 μg/ml

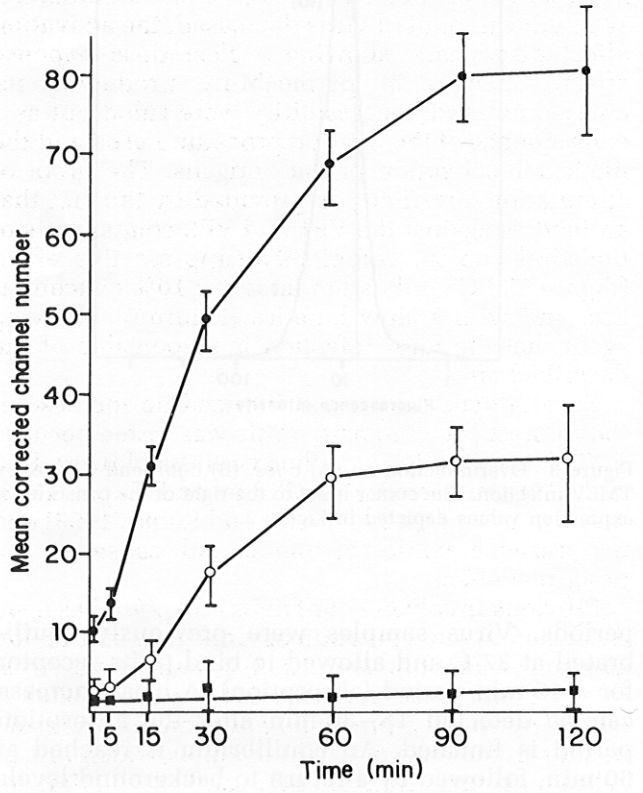

Figure 4 Time course overexpression of c-*fos* (●–●), c-*jun* (○–○) and *ras* (■–■) induced by Theiler's virus. The fluorescence intensity is plotted as mean channel numbers at different times after TMEV infection.

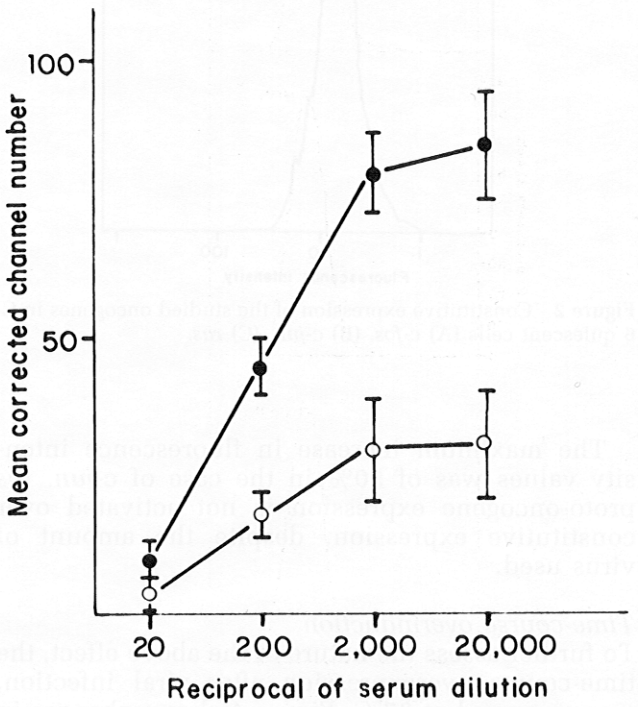

Figure 5 Neutralization of TMEV-dependent c-*fos* (●–●) and c-*jun* (○–○) inducting capacity by a rabbit anti-TMEV antiserum. Results are means of three separate experiments.

of *E coli* LPS showing a moderate increase of *fos/jun* expression (an average of 20 in the mean channel number) with an optimal dose of 100 μg/ml.

Several cytokines as IL-1α, TNFα, IL-6 and IL-2, have been described as potent *fos/jun* inducers in fibroblasts and lymphocytes (Hatakeyama *et al*, 1992; Körholz *et al*, 1992; Kovacs *et al*, 1986; Lin and Vilcek, 1987; Brenner *et al*, 1989). We studied the effect of TFNα, IL-1α, IL-6 and IFN-γ on the expression of the three oncogenes with negative results. Doses of 10–1000 units/ml were unable to induce any oncogene transcription above mean basal expression levels. This was somewhat expected as C-6 does not have receptors for IFN-γ (Rubio and De Felipe, 1991), Il-1α (Rubio, 1994) or TNFα (Aranguez *et al*, 1995).

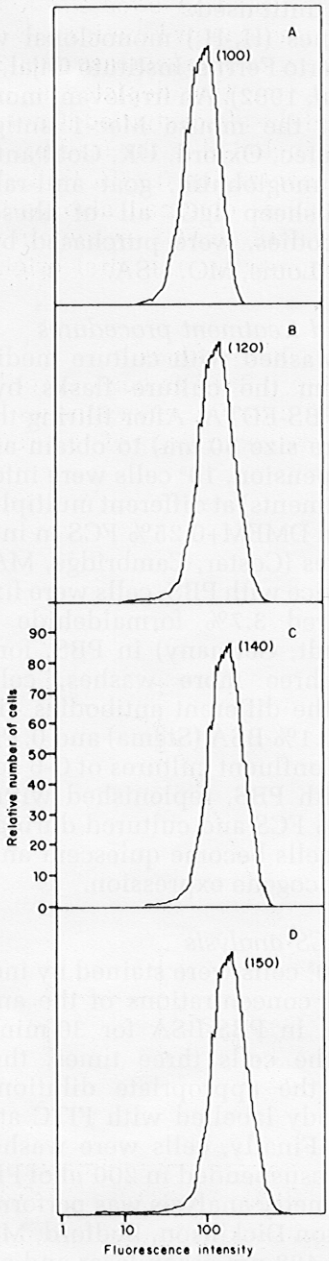

Figure 6 Shift in c-*fos* fluorescence intensity of quiescent C-6 cells induced by incubation with 10% fetal calf serum for 30 (A), 60 (B), 120 (C) and 180 minutes (D).

Discussion

The C-6 cell line has specific receptors for TMEV (Rubio *et al*, 1990) and its binding to them acts as a signal that induces a significant increase in proto-oncogenes c-*fos* and c-*jun* transduction. We used specific antibodies and FACS technology to determine if these proto-oncogenes are activated over basal levels in response to TMEV cell infection. This seems to be an early event in the infectious process.

A basal level of oncogene products was detected in control quiescent cultures, showing that they are constitutively expressed in C-6 cell (Figure 2). This expression was significantly augmented in the case of the members of c-*fos* and c-*jun* family (Figure 3), which encodes nuclear phosphoproteins associated in several transcriptional complexes (Müller, 1986).

The kinetics of stimulation shows that the TMEV stimulatory effect was induced early upon binding to its receptor (Figure 4). A moi of at least 20 (20 PFU/cell) was needed for maximal stimulation. When the amount of virus decreased, the activation effect disappears, showing a clear dose-response effect. Changes in permeability producing increased antibody accessibility were ruled out as a consequence of the fixation procedure used and the nuclear localization of the antigens. The proof of stimulation specificity is provided by the fact that antibodies against the VP1 and VP2 components of the viral capsid, completely suppress this effect (Figure 5). FCS only stimulates at a 10% concentration and with a slow kinetics (Figure 6), showing again that the viral infection is responsible of the described effect.

Several cytokines tested induced no increase in the fluorescence intensity. This was tested because TMEV infection leads to the synthesis of IL-1α, IL-6 and TNFα in astrocytes (Rubio and Capa, 1993; Rubio and Sierra, 1993; Sierra and Rubio, 1993) and its presence could be the actual cause of the described effect.

Previous involvement of c-*fos* and c-*jun* has been described in the regulation of virus gene expression as visna virus (Shih *et al*, 1992) or hepatitis B virus-infected hepato-carcinoma cells (Twu *et al*, 1993). A major fraction of c-*Jun* and c-*Fos* proteins is present in cells in the form of a tight complex which role in cellular signal transduction and regulation of proliferation is well documented (Chiu *et al*, 1988). The potential significance of our findings is that a similar effect could be the first stage of neural cells infection by neurotropic virus.

TMEV persistently infects glioma cell lines. Only a small propotion of cells surviving the lytic infection display cytopathic effects (Patrick *et al*, 1990). To our knowledge, TMEV is the only virus reported to increase expression of early response genes, probably due to the activation of pre-existing transcription factors, in cells of glial lineage. This novel finding of increase in both oncogenes transduction due to TMEV suggests that its products, detected here with specific antibodies, could be associated with the regulation of other genes. This mechanism can be relevant in the infectious mechanisms of neurotropic virus.

Materials and methods

Cells and culture method

The C-6 rat glial tumor cell line (ATCC CCL 107) was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS) and gentamycin (Flow Laboratories, Irvine, UK) at 37°C under 5% CO_2.

Virus and viral proteins

For these studies, a strain of TMEV isolated in 1957 from a feral mouse in Belem, Brazil, called BeAn 8386, was used. After plaque purification and several passages in baby hamster kidney cells (BHK-21), stocks of 1×10^8 plaque-forming units (PFU)/ml were prepared as described (Rozhon *et al*, 1983). Cells were grown at 37°C in DMEM containing 10% FCS and gentamycin. The purification of TMEV virion particles was performed from infected BHK-21 cell monolayers by sucrose and Cs_2SO_4 gradients, as previously described (Lipton and Friedman, 1980). Gradient-purified TMEV was used for infection in all experiments in order to avoid the presence of growth factors.

Individual VP1, VP2 and VP3 virion proteins were purified using a Perkin-Elmer series 3B system and a reverse-phase high-performance liquid chromatography (HPLC) Aquapore RP-300 column from Brownlee Labs (Santa Clara, CA, USA). The viral proteins were allowed to attach to the column matrix in a mixture of 60% formic acid and acetonitrile (80:20). Elution was performed with a linear gradient processed for 60 min, which elevated the percentage of acetonitrile to 80%. Flow rate was adjusted to 1ml/min, and the eluted peaks read at 280 nm. The purity of the individual proteins was checked by sodium dodecyl sulfate-polyacreulamide gel electrophoresis (SDS-PAGE) (Rubio and Cuesta, 1989).

Antibodies

Sheep antibodies anti a c-*jun* sequence common for human and mouse (Ryseck *et al*, 1988), prepared against a synthetic peptide, were obtained from Cambridge Research Biochemicals, Northwich, UK. Sheep anti c-*fos* oncoprotein antibodies were from the same source. A monoclonal antibody nominated Y13-259, that reacts with the P21 product of all three *ras* genes (H, K and N-ras) (Furth *et al*, 1982) was gently provided by Dr Angel Pellicer, Department of Pathology, NYU, New York, USA).

An anti TMEV virus antiserum was produced in New Zealand white rabbits by subcutaneous and intramuscular injections in multiple sites. Four injections containing 300 μg of purified BeAn 8386 virus emulsified with complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) were given at 2-week intervals. The rabbits were bled 10 days after the last booster, and serum was frozen at −20°C until used.

An anti histones (H_1-H_2) monoclonal was provided by Dr. Alberto Ferrus, Instituto Cajal, Madrid, Spain (Fujita *et al*, 1982). An irrelevant monoclonal antibody against the mouse Mac-1 antigen was provided by Serotec, Oxford, UK. Goat anti-mouse polyvalent immunoglobulin, goat anti-rabbit IgG and rabbit anti-sheep IgG, all of these FITC-conjugated antibodies, were purchased by Sigma Chemical Co, St. Louis, MO, USA.

Cell infection and treatment procedures

C-6 cells were washed with culture medium and resuspended from the culture flasks by gently pippeting with PBS-EDTA. After filtring through a nylon mesh (pore size 50 μm) to obtain an homogeneous cell suspension, 10^6 cells were infected (or subjected to treatments) at different multiplicities of infection (moi) in DMEM+0.25% FCS in individual 5 ml culture tubes (Costar, Cambridge, MA, USA). After washing twice with PBS, cells were fixed with a freshly prepared 3.7% formaldehide solution (Merck, Darmstadt, Germany) in PBS, for 30 min at 4°C. After three more washes, cells were incubated with the different antibodies diluted in PBS containing 0.1% BSA (Sigma) and 0.1% Triton X-100 (Merck). Confluent cultures of C-6 cells were washed once with PBS, replenished with DMEM containing 0.25% FCS and cultured during 48 h in order to let the cells become quiescent and obtain basal levels of oncogene expression.

Staining and FACS analysis

Aliquots of 5×10^5 cells were stained by incubating them in optimal concentrations of the antibodies described above, in PBS-BSA for 30 min at 4°C. After washing the cells three times, they were incubated with the appropriate dilution of the secondary antibody labelled with FITC at 4°C for another 30 min. Finally, cells were washed three more times and resuspended in 200 μl of PBS. One-colour flow cytometry analysis was performed on a FACStar^{plus} (Becton Dickinson, Bedford, MA, USA) equipped with a 488 nm argon laser and a 599 nm dye laser. Data from 10 000 to 50 000 cells were collected and analyzed. Computer-assisted data analysis of results was performed on a MicroVAX

computer with FACS/DESK software. Residual dead cells were gated out using side scatter channels and propidium iodide staining at the time of data collection. Fluorescence intensity of individual cells was measured as relative fluorescence units. For negative controls, unstained cells and/or cells stained with irrelevant antibodies were used. Establishment of the gate was based on the staining profiles of the negative control.

Recombinant cytokines and lectines
Mouse recombinant IL-1α was purchased from Genzyme, Cambridge, MA, USA and rIFN-γ of murine origin was from Holland Biotechnology, Leiden, The Netherlands. Recombinant murine Tumor Necrotic Factor-α (TFNα) was purchased from Innogenetics, Antwerp, Belgium. Human rIL-6 was from Boehringer Mannheim, Germany. rIL-2 was obtained from Ortho Pharmaceutical Corp. Raritan, NJ, USA. *E coli* lipopolysaccharide (LPS) and cyclohexamide were obtained from Sigma.

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