Cooperation of oncogenes in cell transformation and sensitization to killing by the parvovirus minute virus of mice

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The established line of normal Fisher rat fibroblasts (FR3T3) is naturally resistant to the parvovirus minute virus of mice (MVM), and was used as a model system to study the influence of stepwise transformation on the susceptibility of cells to this virus. When transformed with genes encoding the class I nuclear oncoproteins large T antigen of polyomavirus (PyLT) or v-myc, cells retained a normal appearance, but acquired some ability to form colonies in soft agar. On the other hand, the class II transforming oncogenes encoding the middle T antigen of polyomavirus (PyMT) and c-Ha-ras-1 induced both morphological alterations and a high capacity for anchorage-independent growth in transfected cells. The concomitant expression of oncogenes from both classes (PyLT+PyMT; v-myc+c-Ha-ras-1) induced a supertransformed phenotype characterized by the piling-up of cells into poorly adherent foci, even in low density cultures. The progressive transformation of this cellular system was found to coincide with a gradual increase in its susceptibility to MVMp (MVM prototype strain) infection. Compared to parental cells, class I, class II and double transformants proved to be sensitized to killing by MVMp to a low, moderate and large extent, respectively. Thus, oncogenes from different functional classes appeared to cooperate in the responsiveness of cells to parvovirus attack. Interestingly, this cooperation exacerbated both the killing of infected cells and their capacity to produce viral non-structural (NS) proteins, in agreement with the reported cytotoxic activity of NS polypeptides. Therefore, in this system, parameters of the parvovirus life cycle may serve as indications of the overall progression of the transformation process.

Introduction

Parvoviruses are small, non-enveloped, nuclear- replicating viruses with a linear ssDNA genome of about 5000 nucleotides. The low coding capacity of parvoviruses implies that they rely strongly upon exogenous helper factors for their replication. In the case of autonomous parvoviruses like the minute virus of mice (MVM), these factors are provided by the host cell and are expressed as a function of cell proliferation and differentiation (Cotmore & Tattersall, 1987). Often isolated from tumour tissues, tumour cell lines or stocks of oncogenic viruses (Siegl, 1984), parvoviruses were suspected of being oncogenic. Nevertheless, they proved to be devoid of such an activity and, on the contrary, to inhibit both spontaneous and induced cancers in laboratory animals (reviewed in Rommelaere & Cornelis, 1991). Therefore, the frequent association of parvoviruses with tumours is likely to reflect an opportunistic relationship instead of a causal one (Rommelaere & Tattersall, 1990). This oncotropism is consistent with the fact that a number of neoplastic or transformed cells cultivated in vitro are permissive to the lytic parvovirus life cycle, whereas normal cells generally resist virus attack. This sensitization of transformed cells correlates with a stimulation of paroviral gene expression, suggesting that some viral proteins may be cytotoxic (Rhode, 1987; Osawa et al., 1988; Cornelis et al., 1988b; van Hille et al., 1989; Brandenburger et al., 1990). It has been demonstrated recently that the intracellular accumulation of MVM-encoded regulatory non-structural (NS) proteins is toxic for neoplastic human cells (Caillet-Fauquet et al., 1990).

It has been shown that tumour viruses, cellular or viral oncogenes, and chemical or physical carcinogens may sensitize normally resistant cells to parvovirus-induced killing (Moussset & Rommelaere, 1982; Moussset et al., 1986; Cornelis et al., 1988a; van Hille et al., 1989; Salomé et al., 1990; Guetta et al., 1990). However, it is unclear whether cell sensitization to parvoviruses correlates with the expression of a specific facet(s) of the transformed phenotype. We have shown previously that abortive transformation induced by infection with the tumour virus simian virus 40 or by treatment with the tumour promoter TPA is not sufficient to sensitize mouse cells to killing by MVMp (prototype strain of MVM) (Moussset & Rommelaere, 1988). In this work, we took
advantage of the fact that in vitro cell transformation can be achieved in a stepwise fashion by using cooperating oncogenes (Cuzin, 1984). This property is related to the somewhat artificial grouping of oncogenes into functional classes I and II, depending on the primary effect (immortalization versus transformation) and localization (nucleus versus cytoplasm or plasma membrane) of corresponding oncoproteins (Hunter, 1991). Two prototype class I [polyomavirus large T (PyLT) and v-myc] and class II [polyomavirus middle T (PyMT) and c-Ha-ras-1] oncogenes were used, on the basis of their previously reported ability to act in cooperation in neoplastic transformation of normal cells (Ruley, 1990). Consistently, we found that PyLT or v-myc caused little phenotypic alteration in the established but otherwise normal rat fibroblast line FR3T3 grown in liquid medium. However, these oncogenes conferred on corresponding PyMT- or c-Ha-ras-1-induced morphological transformants the capacity to pile up into poorly adherent foci, even at low cell density. All single and double transformants achieved a greater cloning efficiency in semi-solid medium to a certain extent. Therefore, this cellular system offered a spectrum of gradual phenotypic alterations to be compared with the susceptibility to MVMp infection. Our results indicate that expression of the class I or class II oncogenes studied resulted in enhanced killing of FR3T3 cells by MVMp, although the latter oncogenes proved to be more efficient. The cooperation of both classes of oncogenes at the level of cell transformation is reflected in a modest synergism of their sensitization of cells to MVMp infection. It is concluded that, in this system, sensitivity to MVMp may be indicative of the progression of the transformation process rather than correlated with a specific step.

**Methods**

*Cells, virus and plasmids.* The established line of Fisher rat fibroblasts FR3T3 (Seif & Cuzin, 1977), its FR4 subclone (Guetta et al., 1990) and corresponding transformed derivatives were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (FCS) and 1% sodium pyruvate. The mouse cell line A9 was cultivated in Eagle's MEM supplemented with 5% FCS. MVMp was propagated in A9 cells and purified according to Tattersall et al. (1976).

The transformed cell lines FRLT1, FRMCI4 and FREJ4 have been described previously by Rassoulzadegan et al. (1982), Salomé et al. (1990) and van Hille et al. (1989), respectively. FR4 derivatives transformed by PyMT (FRMT) or both PyMT and PyLT (FRMLET) were isolated as genetin (0-5 mg/ml)-resistant foci of morphologically altered cells, after cotransfection with plasmid pSV2neo (Southern & Berg, 1982) and pPyMT1 (Treisman et al., 1981) or pPyMLT97 (Gelinas et al., 1989). FRMCEJ clones were obtained by transfection of FRMC14 cells with plasmid pSVneoEJ, which carries the c-Ha-ras-1 oncogene from the human bladder carcinoma cell line EJ (McKay et al., 1986), followed by the selection of genetin (0-5 mg/ml)-resistant colonies. Transfections were carried out by the calcium phosphate precipitation technique described by Brandenburger et al. (1990).

*Detection of oncogenes.* After cell lysis, oncogenes were immunoprecipitated (Salomé et al., 1990) with either the ras-specific monoclonal antibody Y11-259 (Oncogene Science) or polyclonal antibodies that recognized both PyMT and PyLT antigens, obtained by intraperitoneal injection of polyomavirus-transformed PyB4A cells into BN rats. Oncogenes were labelled either in vitro by prior incubation of cells for 1 h (PyLT) or 18 h (c-Ha-ras-1) with [125I]methionine (200 to 500 µCi/106 cells, 800 Ci/m mole; Amersham), or after immunoprecipitation (PyMT) by an in vitro kinase reaction in the presence of [35S]methionine (200 to 500 µCi/ml, 800 Ci/mol; Amersham) according to the method of Gelinas et al. (1989). Proteins were fractionated by 7-5 to 15% SDS–PAGE and visualized by autoradiography. The immunodetection of the v-myc protein in this system has been reported previously (Salomé et al., 1990).

*Clonogenicity in semi-solid medium.* For the measurement of anchorage-independent growth, suspensions of 103 to 105 cells in 3 ml Dulbecco's modified MEM containing 10% FCS and 0-33% agar were seeded onto 60 mm diameter dishes containing 7 ml of the same medium. Colonies were counted by microscopic examination between 3 and 5 weeks after plating.

*Parvovirus cytotoxicity.* Parvovirus-induced cell killing was determined by clonal growth assays, as described by Mousset & Rommelaere (1988). Between 200 and 1000 cells were plated in 60 mm plastic dishes and allowed to form colonies which were stained after 10 days. The plating efficiency of all cell lines ranged from 80 to 100%. Cell survival was calculated from the number of colonies formed by MVMp-infected cells and expressed as a percentage of those formed by mock-infected cells. The m.o.i. was defined as the number of p.f.u. virus inoculated per cell.

*Parvovirus life cycle parameters.* Total viral DNA was measured by hybridization using dispersed cell assays as described previously (Mousset & Rommelaere, 1988). Viral DNA amplification was calculated as the ratio of cell-associated viral DNA at 30 h to that at 2 h after infection at an m.o.i. of 1 p.f.u./cell.

*For the detection of the viral NS-1 protein, cultures were infected at an m.o.i. of 2 p.f.u./cell, incubated for 18 h and further labelled for 1 h with [35S]methionine (200 to 500 µCi/ml, 800 Ci/mol; Amersham). Cells were lysed and NS-1 protein was immunoprecipitated with a monospecific rabbit antiserum raised against a bacterial fusion protein which contains an MVMp NS-1 protein-specific amino acid sequence (Cornelis et al., 1988b). Proteins were fractionated by 10% SDS–PAGE and visualized by fluorography.

**Results**

*Characterization of the cell lines.*

Table 1 summarizes the properties of the cells used in this work. FR3T3 and its subclone FR4 are established lines of normal rat fibroblasts which exhibit stringent contact inhibition and anchorage-dependent growth. The PyLT- and v-myc-transformed derivatives retained both flat morphology and growth arrest at loose confluence, but could be distinguished from parental cells by their significant, although low (1 to 2%), cloning efficiency in soft agar. The PyMT and c-Ha-ras-1 transformants were characterized by their small and rounded appearance,
Oncogene-promoted cell killing by MVMp

Table 1. Phenotypic properties of the FR3T3 cell line and its oncogene-transformed derivatives*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Oncogene alteration</th>
<th>Saturation density (cells × 10^-3)</th>
<th>Clustered efficiency in soft agar (%)</th>
<th>Cloning parameters</th>
<th>MVMp DNA amplification</th>
<th>MVMp cytotoxic effect</th>
</tr>
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<tr>
<td>FR4</td>
<td>-</td>
<td>-</td>
<td>&lt;10^-4</td>
<td>4</td>
<td>92</td>
<td>0.1</td>
</tr>
<tr>
<td>FRLT1</td>
<td>PyLT</td>
<td>9-4</td>
<td>1</td>
<td>12</td>
<td>84</td>
<td>0.2</td>
</tr>
<tr>
<td>FRMT1</td>
<td>PyMT</td>
<td>91</td>
<td>11</td>
<td>51</td>
<td>52</td>
<td>0.65</td>
</tr>
<tr>
<td>FRMT4</td>
<td>PyMT</td>
<td>ND</td>
<td>12</td>
<td>122</td>
<td>20</td>
<td>1.6</td>
</tr>
<tr>
<td>FRMLT1</td>
<td>PyLT + PyMT</td>
<td>150</td>
<td>25</td>
<td>98</td>
<td>24</td>
<td>1.4</td>
</tr>
<tr>
<td>FRMLT4</td>
<td>PyLT + PyMT</td>
<td>ND</td>
<td>13</td>
<td>74</td>
<td>27</td>
<td>1.3</td>
</tr>
<tr>
<td>FR3T3</td>
<td>-</td>
<td>-</td>
<td>&lt;10^-4</td>
<td>11</td>
<td>89</td>
<td>0.1</td>
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<tr>
<td>FRMC14</td>
<td>v-myc</td>
<td>9-1</td>
<td>2</td>
<td>10</td>
<td>65</td>
<td>0.4</td>
</tr>
<tr>
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<td>110</td>
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<tr>
<td>FRMCEJ1</td>
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<tr>
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<td>v-myc + EJ-ras</td>
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<td>57</td>
<td>14</td>
<td>2</td>
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<tr>
<td>FRMCEJ15</td>
<td>v-myc + EJ-ras</td>
<td>160</td>
<td>52</td>
<td>35</td>
<td>4</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* All values are the average from between three and five experiments.
† Measured at confluence on 60 mm plates.
‡ Ratio of intracellular MVMp DNA content at 30 h to that at 2 h post-infection (m.o.i., 1 p.f.u./cell).
§ Residual colony-forming ability of infected (m.o.i. 10 p.f.u./cell) versus that of mock-infected cells, as calculated from at least 200 surviving colonies. Standard deviations were less than 20%.
¶ Calculated assuming a Poisson distribution.
†† ND, Not determined.

and by their ability to grow to a high saturation density and to form colonies in semi-solid medium with great (up to 55%) efficiency. The double transformants (PyLT + PyMT or v-myc + c-Ha-ras-I) exhibited a 'supertransformed' phenotype, with cells piling up into poorly adherent foci prior to full confluence of the culture (Fig. 1), although their capacity to grow in soft agar was not increased compared to that of single PyMT or c-Ha-ras-I transformants.

The expression of transfected oncogenes was ascertained by oncoprotein immunoprecipitation with specific antibodies, followed by SDS–PAGE. PyLT, c-Ha-ras-I and v-myc proteins were metabolically labelled in vivo prior to immunoprecipitation. Owing to the presence of a major cross-reactive cellular protein of similar Mr, the PyMT protein was labelled in vitro after immunoprecipitation, taking advantage of its phosphorylation by associated tyrosine kinase. This analysis confirmed that the PyLT (Fig. 2a), PyMT (Fig. 2b), c-Ha-ras-I (Fig. 2c) and v-myc (Salomé et al., 1990) oncoproteins were produced by their respective transformants. Interestingly, a fair correlation was observed between the levels of PyMT protein (Fig. 2b) and the relative capacities of the corresponding transformants for anchorage-independent growth (Table 1).

Sensitivity of oncogene-transformed cells to the cytotoxic action of MVMp

The susceptibility of parental lines and transformed clones to the cytotoxic action of MVMp was assessed by measuring their ability to form colonies on plastic after virus infection at a multiplicity of 10 p.f.u./cell. As documented previously (reviewed in Rommelaere & Cornelis, 1991), and confirmed by vital staining and observation of cytolytic effects (data not shown), the inhibition of colony formation could be ascribed to virus-

Fig. 1. Morphological appearance of normal FR3T3 cells and transformed derivatives; phase contrast microscopy of cells in culture. (a) FR3T3, (b) FRMC14, (c) FREJ4 and (d) FRMCEJ15. Bar marker represents 200 μm.
induced cell killing. Survival was determined as the residual clonogenicity of MVMp-infected versus mock-infected cells.

Table 1 shows that the parental FR4 and FR3T3 lines were very resistant to MVMp infection (about 90% survival), whereas oncogene-transformed derivatives displayed a broad spectrum of sensitivities to the virus cytopathic effect. The class I oncogenes PyLT and v-myc sensitized cells to MVMp only poorly (84 and 65% survival, respectively), whereas the class II oncogenes PyMT and c-Ha-ras-1 were more efficient in this respect (52 and 23% survival, respectively). Interestingly, the simultaneous expression of both types of oncogenes (PyLT + PyMT or v-myc + c-Ha-ras-1) resulted in a further increase in MVMp-induced killing of double (compared to either class of single) transformants. Assuming a Poisson distribution, it was calculated that the average number of lethal hits per double transformant was greater than the sum of those inflicted on the corresponding single transformants, suggesting a modest synergistic effect of class I and II oncogenes on sensitization of cells to MVMp.

Oncogene-induced modulation of MVMp replication

To unravel the role of parameters of the MVMp life cycle in cytotoxicity, viral DNA replication and expression were measured in the normal parental cells and in their transformed derivatives.

It has been reported previously that some transformed cells have a greater capacity for replicating parvoviral DNA than their normal progenitors (Mouset et al., 1986; Avalosse et al., 1987; Cornelis et al., 1988a). This prompted us to investigate whether the enhanced toxicity of MVMp for FR3T3 fibroblasts transformed by different oncogenes was associated with a stimulation of viral DNA replication. The amplification of input MVMp DNA was measured by hybridization at 30 h after infection with a multiplicity of 1 p.f.u./cell. As shown in Table 1, FR4 and FR3T3 parental lines as well as PyLT and v-myc transformants sustained only a low level of MVMp DNA synthesis. By contrast, PyMT, c-Ha-ras-1 and double transformants replicated viral DNA very efficiently. However, there was no quantitative correlation between the extent of MVMp DNA amplification and cell killing after infection.

The NS-1 protein of autonomous paroviruses is a multifunctional phosphoprotein, the expression of which has been associated with cell killing (Rhode, 1987; Osawa et al., 1988; Brandenburger et al., 1990; Caillet-Fauquet et al., 1990). Therefore it was of interest to determine whether the varying sensitivity of the transformants tested to MVMp infection was related to the differential expression of this viral polypeptide. The synthesis of NS-1 protein was assessed by metabolic labelling and immunoprecipitation. Fig. 3 shows that the parental cell lines FR4 and FR3T3 produced only minute amounts of this protein after infection. Oncogene-induced transformation was accompanied by an increase in NS-1 production to a small (PyLT), moderate (PyMT, v-myc) or high (c-Ha-ras-1, PyLT + PyMT, v-myc + c-Ha-ras-1) extent. This gradient of NS-1 production (Fig. 3) paralleled that of virus cytotoxicity (Table 1), suggesting that the latter may reflect, at least in part, the capacity of cells for expressing the MVMp NS genes. A similar correlation has been reported recently for a series of related ras-transformed rat fibroblasts (van Hille et al., 1989). Together, these data indicate that various oncogenes or combinations thereof are able to interfere in a distinct and positive way with the control of NS protein production in parovirus-infected cells.
Discussion

Use was made in this work of two pairs of viral or cellular oncogenes belonging to the so-called functional classes I (PyLT and v-myc) and II (PyMT and c-Ha-ras-1), and which act in cooperation to cause the neoplastic conversion of normal cells (Cuzin, 1984; Ruley, 1990). Although the FR3T3 line used as the recipient for oncogene transformation had already acquired one of the class I-associated changes, namely immortality, the combination of either PyLT and PyMT or v-myc and c-Ha-ras-1 had a synergistic transforming action on these cells: double transformants grew in poorly adherent clusters of piled-up cells, in contrast with the parental line and either type of single transformant, which formed regular monolayers.

The double transformants were also most susceptible to MVMp-induced killing. Unlike clustered growth, sensitization to MVMp was detectable to some extent even in the single transformants, and was only slightly greater than additive in the double transformants. Thus it appears that, in this system, sensitivity to MVMp is an indicator of overall cell progression into the transformation process, rather than a marker of a specific step thereof. However, it remains to be determined whether further neoplastic progression beyond stages simulated in vitro can also be distinguished by parvovirus parameters.

Transformed cell lines that were sensitized to MVMp also produced greater amounts of viral DNA and proteins after infection, showing that at least one reason why normal cells resist MVMp consists of their poor ability to replicate the virus. It has been shown previously that in this and a number of other systems normal cells are fully competent in virus uptake (Chen et al., 1986; Cornelis et al., 1988a). Thus transformation appears to up-modulate subsequent intracellular step(s) of the MVMp life cycle. The stimulation of NS protein production did not parallel that of viral DNA replication, implying a genuine effect of transformation on MVMp gene expression, in addition to its influence on the amplification of viral DNA templates. The activity of promoter P4 programming of the NS transcription unit has recently been reported to be enhanced in ras-transformed, compared with normal, rodent fibroblasts (Spegelaere et al., 1991).

Interestingly, the sensitivity of the different transformants tested to MVMp-induced killing varied with their capacity for NS protein, but not viral DNA, production. This result may be taken together with recent studies showing that the expression of transfected NS genes is cytotoxic (Caillet-Fauquet et al., 1990). Thus, the correlation observed between transformation-associated increases in NS protein synthesis and cell killing may not be fortuitous but indicative of a causal relationship. However, our data do not rule out the up-modulation of parvovirus cytotoxicity by host cell transformation involving additional controls besides that maintained over NS gene expression. Indeed, normal cells appear to resist the forced production of NS protein at levels that kill transformed derivatives (Mousset et al., 1991).

Both class I and class II oncogene-induced transformants exhibited a distinct enhancement of their susceptibility to MVMp expression and cytotoxic activity. There was no simple correlation between the extent of this effect and the display of more conventional transformation traits, such as morphological alterations, reduced growth factor requirements or anchorage-independent growth. The class I and class II oncogenes tested differ in the subcellular location (intra- versus extranuclear) and primary molecular actions (regulation of DNA metabolism versus membrane or cytosolic activities) of the corresponding oncoproteins. However, many cytoplasmic oncoproteins do modulate the expression of cellular genes, including nuclear-acting proto-oncogenes. This is the case with PyMT, which has been shown to induce c-myc expression (Rameh & Armelin, 1991). Thus, oncogenes from different classes participate in interconnected regulatory circuits whose ultimate targets are...
nuclear genes that are controlled at the level of their expression (Hunter, 1991). The cooperation of various oncogenes in neoplastic transformation suggests that the signalling pathways involved are at least partly distinct. This is supported, for instance, by the fact that some transcription factors are activated by transforming (class II) but not immortalizing (class I) oncoproteins (Waslyk et al., 1989).

The MVMp early promoter contains several putative binding sites for cellular transcription factors (Bodnar, 1988). Therefore it may not be too surprising that both classes of oncogenes have distinct effects on the expression of parvoviral genes encoding cytotoxic proteins, and cooperate in cell sensitivity to MVMp infection.

The class I oncogenes analysed appeared to be much less efficient than their class II cooperators in sensitizing cells to MVMp infection. It is unknown whether this variation reflects the involvement of distinct cellular effectors of the parvovirus life cycle, or the differential modulation of a common effector. It would be interesting in this respect to determine whether MVMp responsiveness to oncogenes of a given class can be selectively abolished by site-directed mutagenesis of viral promoter sequences.

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