Further Evidence for Deletion of Envelope Glycoprotein (gp69/71)
Sequences in Formation of Moloney-murine Sarcoma Virus

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SUMMARY

Moloney-murine sarcoma virus (S+L- strain of M-MSV) has been non-
productively cloned in murine and non-murine host cells (S+L- cells) and the
expression of Moloney leukaemia virus (M-MuLV) 30000 mol. wt. core protein
(p30) and envelope glycoprotein (gp69/71) were studied by radioimmunoassay.
Antigenic determinants of the M-MuLV p30 were associated with the sarcoma
virus genome in these non-productively transformed cell clones studied, while
the determinants of M-MuLV gp69/71 were not. The absence of envelope-
associated glycoprotein expression in sarcoma virus transformed cells was con-
firmation of biological studies demonstrating that rescued sarcoma virions
acquire envelope-associated properties of host range, neutralization and inter-
ference from rescuing helper virus, and further evidence that the M-MuLV
gp69/71 sequences have been deleted during the formation of the M-MSV.

During the course of these studies, it was also found that S+L- dog cells were
releasing into culture supernatant large amounts of the p30 antigenic determinant,
apparently as a soluble antigen.

INTRODUCTION

Some mammalian type C viruses are known to cause leukaemias, lymphomas and sar-
comas (Gross, 1970; Harvey & East, 1971; Scher et al. 1975; Tooze, 1973). These oncogenic
type C viruses appear to cause disease by two different mechanisms. The first, exemplified
by leukaemias resulting after a long incubation period such as the leukaemia of AKR mice
(Lilly et al. 1975), may occur in vivo by increasing, during replication of these viruses, the
levels of RNA from potentially oncogenic cells or other integrated virus transforming genes
(Peebles et al. 1976b).

The second mechanism by which the mammalian type C viruses cause cancer is by the
formation of replication-defective true deletion mutants which have recombined with
potentially oncogenic information of cell or virus origin (Peebles et al. 1975; Scolnick et al.
1975b; Frankel & Fischinger, 1976). These replication-defective transforming viruses, when
injected into animals, rapidly cause sarcomas or leukaemias (Harvey, 1964; Moloney, 1966;
Gross, 1970; Theilen et al. 1971; Scher et al. 1975). Characteristically, these viruses are
able to enter and transform fibroblasts and other cells but they are defective and unable
thereafter to replicate progeny virions without their associated undeleted replicating non-
transforming helper type C viruses (Huebner et al. 1966; Peebles et al. 1971).
Certain of these defective-transforming viruses have been cloned in non-productively infected heterologous host cells in order to study their nucleic acid sequences and their remaining helper virus structural protein expression in the absence of the associated helper virus (Bassin et al. 1971b; Aaronson et al. 1972; Peebles et al. 1973, 1975; Aaronson et al. 1975; Scolnick et al. 1975a). Previous studies have demonstrated that the best studied of these viruses, the S+L- strain of Moloney-murine sarcoma virus (S+L- M-MSV), apparently lacks the information for virus reverse transcriptase but continues to code for an approx. 60,000 mol. wt. protein precursor (p60) which is cleaved to yield the p30, p15 and p12 proteins of M-MuLV (Oskarsson et al. 1975; Peebles et al. 1976a). The data herein confirm the presence of the p30 determinant in S+L- heterologous host cells by radioimmunoassay and further indicate that the envelope glycoprotein of approx. 70,000 mol. wt. (gp69/71) does not appear to be linked to or expressed by the S+L- M-MSV genome.

METHODS

Cell lines and culture techniques. The origin and culture methods of 3T3FL normal mouse cells, canine kidney cell line MDCK, mink lung cell line, MV-1-Lu, human AV-3 and L-132 cells have been described previously (Bassin et al. 1971a; Peebles et al. 1973, 1975).

Virus antigen preparations of normal, S+L- cells and cell culture supernatants. Approx. 2 x 10^8 normal and S+L- murine and non-murine cells were washed three times with phosphate buffered saline before resuspension in TEN buffer [20 mM-tris-HCl (pH 7-6), 1 mM-sodium EDTA, 100 mM-NaCl], centrifuged at 2000 g for 5 min, recentrifuged after a second wash with TEN buffer, and the cell pellet frozen and stored at -70 °C before assay. Cell culture supernatants were centrifuged at 2000 g for 5 min at 4 °C and clarified again at 10000 g for 20 min at 4 °C.

Virus protein purification, antisera and radioimmunoassays. The major structural protein of Rauscher MuLV (R-MuLV p30) and the virus membrane glycoprotein of Rauscher MuLV (gp69/71) were purified as previously described (Strand & August, 1974). Rabbit anti-FeLV (Theilen) and goat anti-Rauscher MuLV p30 sera were prepared and characterized as previously described (Strand & August, 1974). Quantitative analysis of virus antigens was carried out by competition radioimmunoassay as described by Strand & August (1974). A limiting amount of antiserum sufficient to precipitate approx. 50% of labelled virus antigen was added and the competitive inhibition of binding of labelled antigen by unlabelled virus protein was measured.

RESULTS

Derivation of cells transformed by Moloney-murine sarcoma virus

Moloney-MSV was initially cloned in 3T3FL mouse cells; these transformed cells were termed D-56 (Bassin et al. 1971a). D-56 cells co-cultivated with feline embryo fibroblasts were superinfected with Feline leukaemia virus (FeLV) to obtain an MSV pseudotype [M-MSV(FeLV)] capable of infecting human cells (Peebles et al. 1973). This MSV was cloned in human AV-3 cells and these cells were termed S+L- HuAC1 (Papageorge et al. 1974). The MSV was rescued from S+L- HuAC1 by superinfection with RD-114 virus (McAllister et al. 1972) and was cloned in human L-132 cells with limiting dilution of virus; these cell clones termed S+L- HuLC1 to Cl6 did not appear to contain the RD-114 helper virus (Peebles et al. 1973; Papageorge et al. 1974). This same MSV from S+L- HuAC1 was transferred by superinfection with RD-114 virus and again cloned in mink cells (one clone, S+L- mink, or S+L- MiCl1) and dog cells (four clones, S+L- DoCl1
Fig. 1. Radioimmunoassay of S+L- cells for murine virus p30 antigen. Competition radioimmunoassay used R-MuLV p30 against goat anti-R-Mu-LV p30. Competing antigens were prepared from S+L- dog Cl1 (■), S+L- HuCl1 (□), S+L- HuLC1 (△) and Cl1 (▲), S+L- mink (▽), and normal uninfected mink (▼) and dog (○) cells. Competing purified R-MuLV p30 was used as control (●).

to Cl4). The S+L- HuCl1 cells were also co-cultivated with normal 3T3FL mouse cells, superinfected with M-MuLV, and the MuLV rescued MSV was again cloned back into 3T3FL mouse cells with limiting dilution so as to omit the helper MuLV by isolating non-producer clones S+L- MoCl1 to Cl5.

Analysis of S+L- cells for virus p30

The concentration of virus p30 protein in the different S+L- and control cells was measured by competition radioimmunoassay. The interspecies assay system using purified Rauscher virus p30 as 125I-labelled antigen and anti-FeLV serum (data not shown) measured the interspecies determinants of virus p30 protein in S+L- cells but not in uninfected cells. Alone, these assays did not distinguish the several possible sources for this antigen,
Fig. 2. Expression of virus envelope glycoprotein in S+L- cells. Competition radioimmunoassay used $^{125}$I-labelled R-MuLV gp69/71 against anti-FeLV (Theilen). Competing antigens were prepared as described from normal uninfected cells (a) and MSV-infected S+L- cells (b). (a) Normal 3T3FL mouse cells (□), normal mink cells (■), normal human cells (△), normal dog cells (○) and control purified R-MuLV gp69/71 as positive control (●). (b) S+L- mouse Cl3 (■) and D-56 (▲), S+L- human HuACl1 (□) and HuLCl1 (○) and HuLCl3 (▲), S+L- mink (△), S+L- dog Cl5 (▼).

the murine, feline or RD-114 helper virus or the endogenous viruses of the host cells. In order to distinguish the species of origin of the protein, the cell extracts were tested in a homologous assay system of Rauscher p30 and anti-Rauscher p30 serum; only murine virus protein containing p30 group- or type-specific determinants are detected in this assay system (Strand & August, 1974; Fig. 1). Each of the human and dog S+L- cell clones were positive in this assay system. The mink cells gave minimum displacement which alone could not be interpreted as positive but in the context of the findings with other cells suggests that the antigen in these cells is of murine origin as well. Moreover, S+L- mink cells are flat revertants with little detectable MSV RNA until superinfected with helper virus (Peebles
MSV envelope glycoprotein expression

et al. 1976b); consequently, the slight expression of murine p3o found in these cells is expected. The normal mink and normal dog cells were negative as expected.

Expression of virus envelope glycoprotein

The possible expression of the interspecies antigenic determinants of virus envelope glycoprotein was studied with an assay system containing Rauscher gp69/71 antigen and anti-feline virus serum (Fig. 2). It had previously been found that the interspecies reactivity of the Rauscher glycoprotein in this system was limited and would detect only murine and feline virus glycoproteins (Strand & August, 1974).

A remarkable finding was that the normal 3T3FL cells expressed a high concentration of the glycoprotein, equivalent to that which normally would be found in productively infected cells (1000 ng/ml). This finding indicates the advantage of studying MSV in heterologous host cells presumably lacking the endogenous viruses seen in mouse cells.

The other normal cells showed an apparent low affinity displacement at high protein concentrations. Such displacement with 125I-labelled gp69/71 antigen from concentrated cell or tissue extracts is commonly observed, but because of the apparent low affinity reaction, it is not considered to be positive for virus envelope antigenic determinants.

The S+ L- cells of murine origin were all positive for envelope glycoprotein; as these were all 3T3FL cells, this can be attributed to the normal expression of the virion envelope of an endogenous virus (Nomura et al. 1976), not to an MSV-coded gene product. The displacement curves of the remaining S+ L- cells of dog, human and mink resembled those of normal cells.

Release of virus proteins by S+ L- dog cells

The release of virus proteins by the S+ L- cells, possibly as some type of particle, was studied by analysis of the tissue culture fluid for the presence of p3o antigenic determinants. For example, the endogenous virus genes of the cell could provide the virion envelope and possibly other proteins, whereas the sarcoma virus could code for the p3o and other small mol. wt. protein present in the 65000 mol. wt. precursor polypeptide (Oskarsson et al. 1975). In the case of cells of murine origin, the formation of pseudotype viruses comprised of sarcoma virus genes and proteins of endogenous murine virus is well known (Nomura et al. 1976) and earlier reports of the synthesis of defective particles by S+ L- cells infected by the Moloney-MSV can now be attributed to the formation of such pseudotypes (Bassin et al. 1971b).

In the heterologous assay system (murine 125I-labelled p3o and anti-feline virus serum) no p3o antigenic determinants were detected in supernatant fluids from S+ L- and normal human and mink or normal dog cell clones. In contrast, culture fluids from the S+ L- dog cells showed a high concentration of protein carrying p3o interspecies antigenic determinants (Fig. 3a). The murine identity of this protein was demonstrated by use of the homologous competition assay (Rauscher murine virus p3o and anti-Rauscher murine p3o) specific for type and group antigenic determinants of the protein (Strand & August, 1974; Fig. 3b). The lower affinity and reduced concentration of antigenic determinants are expected for a murine virus other than Rauscher virus in this assay system. It thus appears that the dog S+ L- cells are releasing protein containing the p3o antigenic determinants. The amount of this protein is comparable to that found in the supernatant fluids of RD-114 superinfected MSV producing S+ L- dog cells.

These S+ L- dog cells were then studied by electron microscopy and 3H-uridine banding in sucrose gradients. There was no detectable virus particle production and certainly not of
Fig. 3. Radioimmunoassay for release of virus P3O protein from S+L- cells into culture supernatants. Competition radioimmunoassay of normal and S+L- heterologous host cell culture supernatant fluids using 125I-labelled R-MuLV P3O against anti-FeLV and anti-MuLV P3O. Antigen was assayed by competition radioimmunoassay with (a) the heterologous reaction of R-MuLV P3O with rabbit anti-FeLV and (b) with the homologous reaction of R-MuLV P3O with goat anti-R-MuLV P3O. The supernatants tested were normal dog (△) and S+L- dog (▲) cells, normal mink (□) and S+L- mink (■) cells, and normal human F-49-1 (○) and S+L- human (◇) cells. ●, Standard MuLV P3O.

the magnitude to explain the amount of antigen detected. Consequently, this suggests the release of a soluble p3O antigenic determinant into the supernatant of these S+L- dog cells (Bolognesi & Langlois, 1975).

DISCUSSION

The S+L- strain of M-MSV has been cloned and recloned in non-murine host cells in the absence of detectable replicating helper viruses. These cells have been examined by radioimmunoassay for the expression of remaining MuLV structural proteins, specifically the virus core protein p3O and the envelope glycoprotein gp69/71. In all cases, the p3O determinant remains associated with the MSV genome. The p3O antigenic determinant of S+L- M-MSV has been demonstrated to be part of a larger 60,000 mol. wt. p60 protein precursor (Oskarsson et al. 1975; Van Zaane et al. 1975). These cumulative data are strong evidence indicating that the p60 is stably linked to the transforming gene(s) of S+L- M-MSV (Peebles et al. 1975).

No heterologous host S+L- cell lines had evidence for the murine leukaemia virus gp69/71. Recent work has indicated that the gp69/71 is the virus envelope function that appears to be the primary determinant for host range, interference and neutralization properties of mammalian type C viruses (Hunsmann et al. 1974). Biological studies have demonstrated that the host range, interference and neutralization properties of the S+L- MSV rescued from heterologous host cells are determined not by the MSV genome but by the rescuing helper virus (Peebles et al. 1973, 1975; Hellman et al. 1974; Papageorge et al. 1974). Consequently, the absence of murine gp69/71 in all of these clonally derived heterologous host S+L- cells is consistent with the biological data and both are very good evidence that the gp69/71 gene has been deleted in the formation of this MSV genome. The
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virus polymerase gene has also been deleted in S + L - M-MSV (Peebles et al. 1975, 1976a). The deletion of both of these functions during the formation of the sarcoma virus genome would indicate that these genes may be closely linked as previously suggested (Peebles et al. 1975). Consequently, it appears that the parental replicating Moloney leukaemia virus undergoes both deletion in the polymerase and gp69/71 genes and recombines with transforming sequences to become a replication-defective but transforming virus, S + L - M-MSV (Scolnick et al. 1975b; Frankel & Fischinger, 1976).

Cumulative biological data from studies of the murine (Peebles et al. 1975, 1976a; Parks et al. 1976) and primate (Aaronson et al. 1975) transforming viruses indicate common patterns in the functions deleted and in the remaining leukaemia helper virus proteins expressed by sarcoma viruses isolated in the absence of the helper virus. These common deletion patterns are strong evidence (1) for a genome organization common to all replicating mammalian type C viruses and (2) for a common mechanism in all mammalian species for generation of transforming viruses from parental helper viruses. This mechanism must also involve recombination with new transforming sequences presumably of cell or other virus origin. The resultant deleted virus is defective in its ability to produce progeny in the absence of helper virus, but is now able to transform cells rapidly.

During the course of these studies, it was also noted that S + L - dog cells were unique in that they released large amounts of a soluble p30 antigenic determinant into their culture supernatant fluids in the absence of detectable virus particle production and under optimal conditions for cell viability. Although Bolognesi & Langlois (1975) have described the release of a soluble virus gp71 from cells, this is the first example of the in vitro release of the major structural core virus protein in a soluble form in the absence of virus particle production.

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