Studies on Murine Sarcoma Virus
III. Synthesis of the Viral Antigens in Cells of Various Species (36301)

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It was shown in a previous publication (1) that, by use of appropriate immune sera, the group-specific (GS) and virus envelope (V) antigens of murine C-type viruses could be detected by the immunofluorescence (IF) technique in chronically infected mouse and rat cell lines and in a recently infected continuous mouse cell line.

The aims of the present paper are: (i) to describe, with emphasis on its early aspects, the synthesis of GS and V antigens in mouse cells of different genotypes, recently infected with mouse sarcoma virus, strain Moloney (M-MSV); and (ii) to report the results of attempts to promote the synthesis of these antigens in other host cells of animal (rat, hamster, and chicken) or human origin.

Material and Methods. Cells. The cell types used in the present work are listed in Table I. Primary cultures of mouse, rat, and hamster embryo (ME, RE, and HE) cells were prepared from 11- to 16-day-old embryos and chick embryo (CE) cells from 11-day-old embryos. Care was taken to ensure age uniformity in a given experiment. Secondary cultures were used for infection. All the cells were grown in Eagle's minimum essential medium supplemented with 10% calf serum (MEM-10), arginine, and antibiotics (penicillin, streptomycin).

Virus. M-MSV preparations were obtained from mouse tumor extracts or from the growth medium of the 78 Al cell line (2), in the latter case after precipitation by 8% (w/v) polyethylene glycol (PEG). Murine leukemia virus, strain Moloney (M-MuLV) was prepared from the viremic plasma of infected BALB/c mice. The virus suspensions were concentrated by spinning at 56, 000*g* for 90 min (M-MSV tumor extracts and M-MuLV-containing plasmas) or at 105, 000*g* for 30 min (PEG-precipitated M-MSV) in the Spinco model L ultracentrifuge. In some experiments, the virus stocks were further purified by sedimentation in a 15–60% (w/v) sucrose buoyant density gradient. The method used for determining the titer of M-MSV as focus-forming units (FFU) was as described by Hartley and Rowe (3), except that maintenance medium was added after a 90-min incubation with 1 ml of virus dilution. All the virus stocks used in the present study gave titration curves deviating from "one-hit" focus titration patterns, thus reflecting the presence of defective M-MSV virions (3, 4).

Sera and immunofluorescence. The sera used for detection of GS and V antigens, and the technique utilized for IF reactions were described previously (1). Anscochrome (500 ASA) films were used for microphotographs.

Experimental Methods. Leighton tubes containing coverslips were seeded with 2×10^5 cells and 50-ml Falcon plastic flasks with 7×10^5 cells. These cultures were infected at the desired multiplicity 18–24 hr after planting. Adsorption was carried on for 90 min at 37° and was followed by 2 washings with MEM-10, after which maintenance medium (MEM-5) was added. Infected and uninfected control cells were examined for morphological alterations and processed for IF at regular intervals after infection. The cells grown on coverslips were washed 2× with Dulbecco's phosphate buffered saline (PBS), allowed to dry and fixed in acetone

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TABLE I. List of the Cell Types Tested.

Mouse embryo cells
BALB/c
C57BL/6
A/Sn
AKR
C3H/eB
C3H/He
DBA/2
Mouse cell lines
BALB/c 3T3
BALB/C CL-1 (8828)
Rat (W/Fu), hamster, and chicken embryo cells
Human diploid cell lines (1295, WI-38)

for 10 min at room temperature. The cells infected in flasks were trypsinized (0.25% trypsin, Difco), washed 2× by centrifugation, spread on coverslips, allowed to dry, and processed as above. Fixed coverslips were then stored at −20° in screw-capped vials until tested.

In some experiments, the cells were exposed to 50 μg/ml of diethylaminoethyl-dextran (DEAE-D) (2×10^6 daltons, Sigma Chemical Co) for 30 min in MEM-10, washed 2× with PBS, and infected. When the observation period was to exceed 5–6 days, 250-ml Falcon flasks seeded with 2×10^6 cells were used for infection. The cells were subsequently subcultured at regular intervals and part of them was seeded or spread on coverslips at every subculture for IF reactions.

Results. Mouse cells. Most of the experiments recorded in this paper were done at a multiplicity of infection of 1:20 (Leighton tubes) or 1:35 (50-ml flasks), these multiplicities being calculated from the ratio of FFU to the number of cells without taking into account the number of M-MuLV virions also present in the virus stocks used (3, 4), with a ratio of leukemia virion to sarcoma virion of 10^2 to 10^3 (3). Infection with M-MSV at these multiplicities was constantly followed by gross modification of the monolayers at 24 hr. All the cells in the cultures appeared to have sharper outlines and increased refringence and tended to separate from each other. At 48 hr many round cells were present, some of them detaching very readily from the monolayer. However, they were always fewer than spindle cells.

The time course of appearance of GS antigen in infected cultures, as determined from the results of a representative experiment, are shown in Fig. 1. If some synthesis of the antigen could be detected as early as 12 hr (Fig. 2a), a few cells only could be recognized as undoubtedly positive at that time, and accurate counting could be done only at later hours (Fig. 2b). At 24 hr all the mouse cells examined, regardless of their genotype or origin (continuous line or ME cells), were found positive, the percentage of fluorescent cells lying between 4 and 8%. About 20% of the cells were positive at 48 hr; this proportion increased slightly or remained constant at 72 hr, according to the experiment. In some cases the percentage of fluorescent cells was very low at 24 hr, and increased only between 48 and 72 hr.

The time course of appearance of V antigen was found, on the whole, to parallel that of GS antigen. However, minor differences were regularly observed in the exact timing and in the respective percentages of fluorescent cells. After 72 hr, the percentage differences were usually more pronounced and tended to favor the proportion of GS antigen-synthesizing cells. However, the proportion of V antigen-containing cells was concomitantly found higher in the cells floating in the growth medium.

The staining obtained with GS antiserum did not, as already described for CL-1 cells (1), involve the nucleus (Fig. 2c) and seemed to move from the perinuclear area (Fig. 2c) to the periphery of the cell after 48 hr (Fig. 2d). At early hours, V antigen was very similar in appearance to GS antigen (Fig. 2c and e) and the distinction between the two antigens rested rather on the nature of the respective serum and the slightly different percentages of positive cells. At 48 hr and later, on the contrary, the aspect of the two antigens was strikingly different (Fig. 2d and f), as already reported for CL-1 cells (1).

Even at the multiplicities employed in the present work, although all the cells seemed to be altered in the microscope, IF examina-

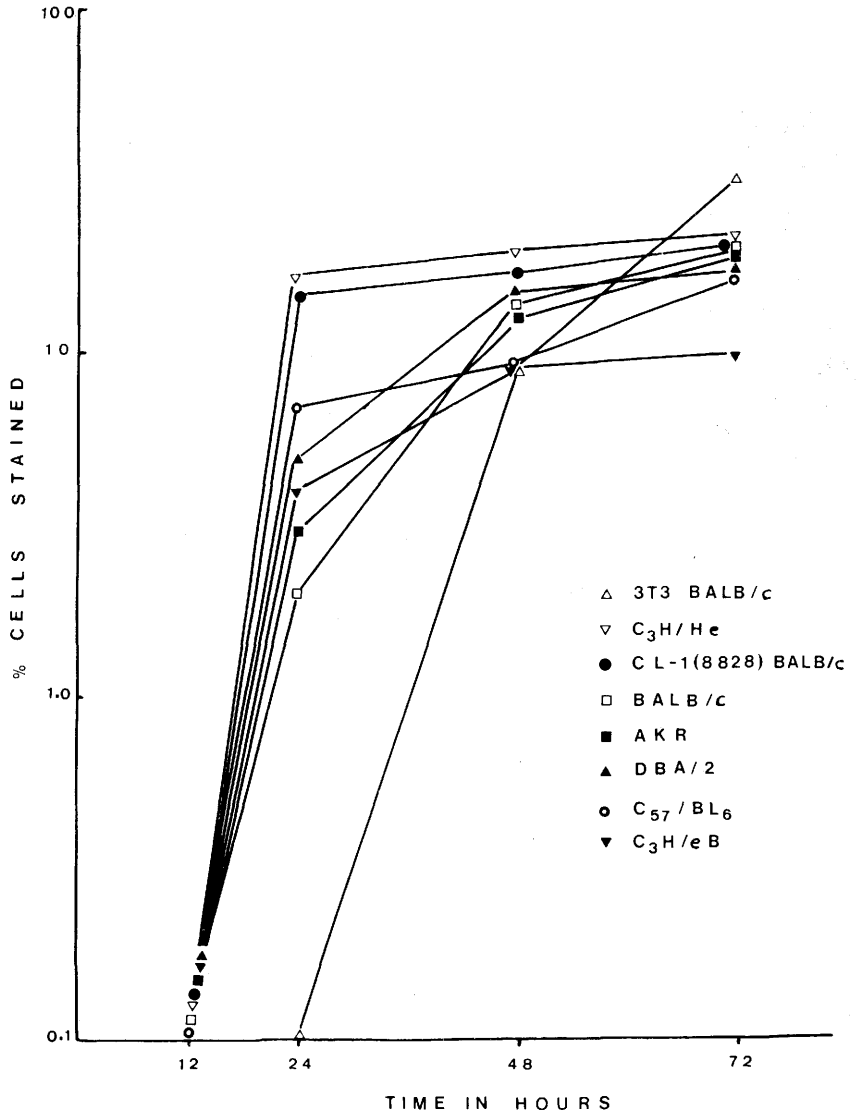
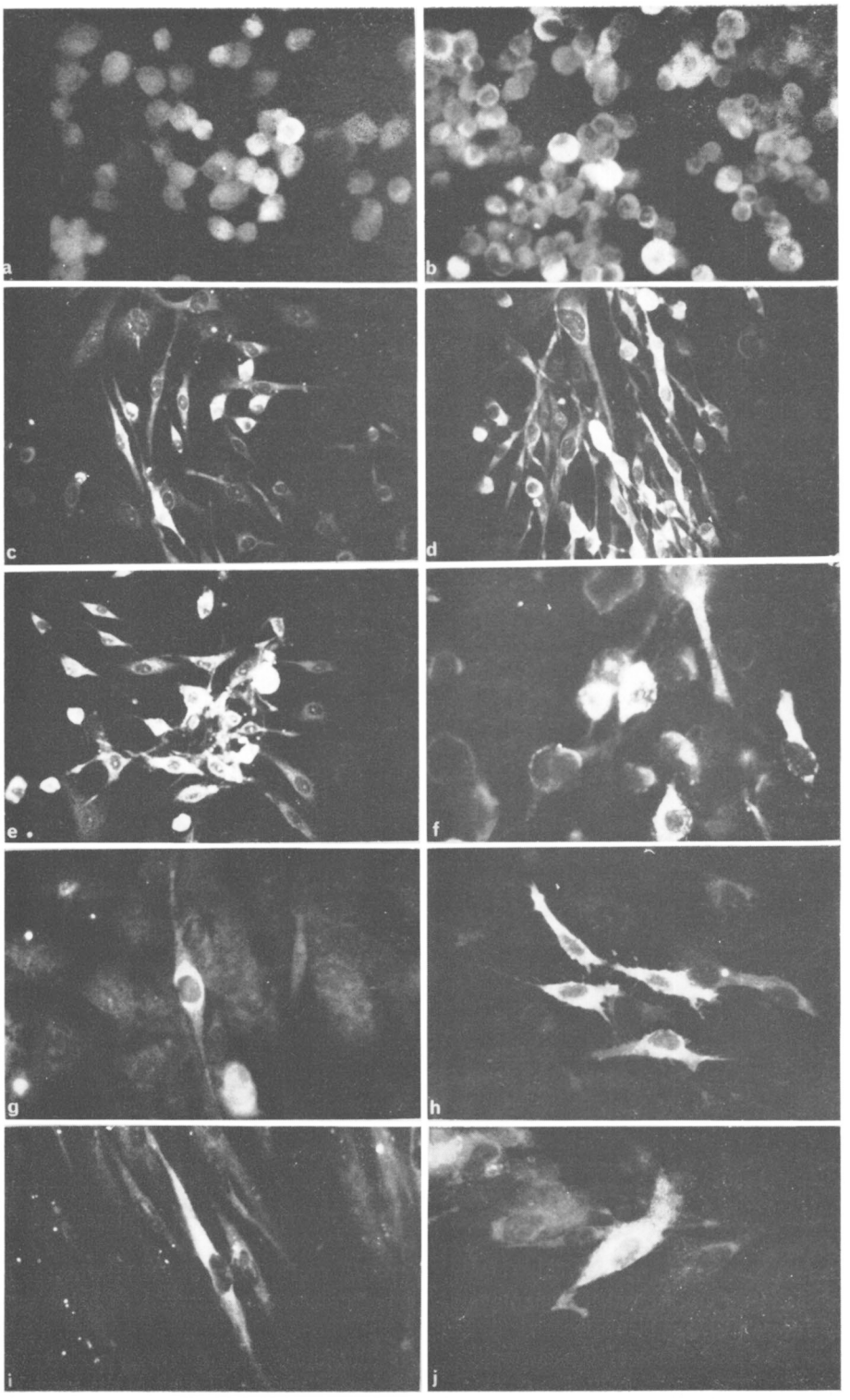


FIG. 1. The percentage of mouse cells of various genotypes containing GS antigen, as detected by immunofluorescence, is plotted as a function of time after infection with MSV at a multiplicity of 1:35 (calculated as focus-forming units). The various cell types were inoculated with the same virus batch and samples were trypsinized into smears at the times indicated.

tions showed that only a fraction of the cells were stained. This fraction was estimated as a percentage of stained cells from countings most conveniently performed on cell smears. When the cells were observed as monolayers, some of the positive cells appeared to be gathered in localized areas of the coverslips; whereas others were single (Fig. 2b, c, d). In a few experiments, other multiplicities were utilized. Higher multiplicities resulted in an increased percentage of fluorescent cells in cell smears (50% of the cells positive at 72 hr at a multiplicity of 1:2); whereas a regular decrease was observed with increasing virus dilutions. Fluorescent cells were present at dilutions where cytopathic alteration was no longer visible. However, the very low percentages obtained at high dilutions render the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determination of an end point rather inaccurate. Infection with M-MuLV gave similar results as regards the timing of the synthesis of the antigens and the staining pattern. With both viruses the proportion of stained cells in the subcultures regularly decreased to lower percentages.

Rat cells. No alteration of the monolayer was observed in the present experiments. However, the time course of appearance of both GS and V antigens closely followed the pattern found with mouse cells (Fig. 2g and h).

Hamster cells. No synthesis of murine C-type virus GS and V antigens could be detected in infected hamster cells, even after pretreatment of the cultures with DEAE-D and utilization of a high multiplicity (1 FFU/cell). The infected cultures were continued in consecutive passages and occasionally refed with fresh HE cells, without any success.

Chick cells. Nonspecific staining was constantly present unless density gradient-purified virus was used. However, negative results were also obtained with CE cells, even after DEAE-D treatment and prolonged culture of the infected cells.

Human cells. Infection of the two diploid cell lines with M-MSV at a multiplicity of infection of 1:10 failed to produce any change in the cell culture during the first week postinfection (pi). Similarly, all electron microscope (EM) and IF observations only gave negative results. However, morphologic conversion of the monolayer was obvious 3 days after the first subculture, *i.e.*, 10 days pi, together with abundant production of virus particles in the EM and appearance of the viral antigens in IF. It should be pointed out that pretreatment of the cells with DEAE-D and infection with tissue culture-grown M-MSV concentrated by PEG precipitation were critical for obtaining positive results. If either of these conditions was missing, no (or extremely poor) virus replication seemed to take place.

The morphologic alteration of the human diploid cell lines infected with M-MSV was strikingly different from that observed in mouse cells. The conversion was diffuse, in that no foci were recognized at the multiplicity employed, the altered cells being interspersed with a monolayer of normal cells. The main feature of the altered cells, which consisted almost exclusively of spindle cells, was their high refringence.

The major difference in the IF aspect of the viral antigens was also the predominance of spindle cells (Fig. 2i and j). In addition it was often found that the GS antigen had a granular rather than homogeneous appearance, the granules being extremely dense and sometimes very distinct.

The morphologic alteration of the culture had disappeared with the second subculture; whereas in consecutive passages the EM and IF examinations constantly yielded positive results. The percentage of fluorescent cells was generally around 20% in the first subculture. However, this percentage steadily decreased in the course of time, lying for example between 0.5 and 3% by passage 20. The late percentages were never found to exceed those found in the early passages.

Discussion. The present results confirm and extend those previously obtained with the continuous mouse cell line CL-1 (1), showing that the synthesis of the GS and V antigens of M-MSV can be detected as early as 12–15 hr pi. Earlier investigations along this line, performed with rabbit immune serum, showed that the antigens of Friend leukemia virus (F-MuLV) were detectable 3 days pi, at the earliest, and that the fluorescence was still localized in the perinuclear areas of the cell at 5 days, without any cell surface fluorescence even appearing (5). Studies with Rauscher virus (R-MuLV) and M-MuLV, using the same type of serum,

FIG. 2. Indirect immunofluorescence staining of M-MSV-infected mouse, rat, and human cells with rat serum to group-specific (GS) antigen or mouse serum to virus envelope (V) antigen. (a,b) smears; (c through j) monolayers. Magnification: (a,b,c) 175× (d,e) 437× (f through j) 700×. (a,c) mouse embryo cells, GS 12 hr postinfection (pi) (b,d) 72 hr pi; (e) V 24 pi; (f) V 72 hr pi; (g) W/Fu rat embryo cells, GS 24 hr pi; (h) 48 hr pi; (i) human diploid cell line 1295; GS 12 days pi; (j) V.

similarly revealed that an interval of 5 and 11 days, respectively, was necessary (6). In more recent studies, using rat immune sera with both GS and V reactivity, an interval of 7 days was found for Gross virus (G-MuLV), the fluorescence being maximal at 10 days (7).

The genotype of the host cell was not consistently found to influence the timing and the proportion of fluorescent cells. This suggests that the reason for the discrepancies between the present and the other studies lies rather in the type of serum used. An additional reason might be the utilization of murine leukemia virus strains with different passage histories. It should be pointed out that M-MSV and M-MuLV, at least, behaved similarly in the present studies.

In this context, the respective role of M-MSV and M-MuLV virions both present in M-MSV stocks, cannot be assessed from the present data. It must be borne in mind that the multiplicities employed were calculated from the ratio of the number of FFU to the number of cells and that units of focus formation and units of antigen induction are not correlated. As percentage end points have been found unsuitable for titration purposes, studies with other techniques are in progress in our laboratory.

Viral infection appeared to spread very slowly in the cultures and some findings even suggested that the increase in fluorescent cells with time might result, at least in the first few days, from cellular division rather than from actual spreading from cell to cell. However, studies with synchronized cell clones would be more conclusive.

The finding that, at a multiplicity of infection of 1:35, the cytopathic effect observed in mouse cells at 24 hr apparently involved all the cells of the monolayer, whereas only 20% of these were actively engaged in viral antigen synthesis, suggests that this early cytopathic effect might be relatively independent of virus replication. It is known from past experience in this laboratory that rat cells can be transformed by M-MSV (2). However, no cytopathic effect was observed in the present experiments although the synthesis of the viral antigens was proceeding as effectively as in mouse cells. This lends further support to the idea that cellular alteration and viral replication are indeed two independent consequences of M-MSV infection. The hypothesis that only M-MuLV was being replicated must also be considered but cannot be ruled out from the present results.

The negative results obtained with chick cells are not surprising. Various strains of Rous sarcoma virus (RSV) have been reported to induce tumors in mammals and to transform mammalian cells *in vitro*. Up to the present time, no mammalian tumor virus has been reported to induce tumors in birds. However, since the replication of RSV in mammalian cells does not seem to go beyond the synthesis of avian C-type virus GS antigen, it was deemed of interest to see whether murine GS antigen might be produced in MSV-M infected chicken cells.

The relationships between hamster cells and the various strains of MSV are rather complex, but recent evidence (8) strongly suggests that the "hamster-tropic" C-type virus particles shed by most, if not all, tumor cell lines induced by MSV is indeed an indigenous C-type virus of the hamster. This would account for the puzzling contrast between the presence of these particles and the failure to detect GS antigen reactivity in the cells (9), even by IF (1). The studies reported here were initiated before these results were published to determine if some synthesis of the murine GS antigen could be induced in hamster cells recently infected by M-MSV. The results extend those reported by Ting and Bader (10) in which, in contrast to the Harvey strain of MSV (H-MSV) and a R-MuLV pseudotype, M-MSV failed to transform, and replicate in, infected HE cells.

As previously reported from this laboratory, M-MSV can replicate in human cells (11). In the present studies it was shown that, in addition to being transformed after infection with M-MSV, the two diploid cell lines tested were found to support the synthesis of both GS and V murine antigens for over 20 consecutive passages. This result is important in view of the findings mentioned

above concerning hamster-tropic viruses. The eventual emergence of such antigenically unrelated viruses, granted that it can occur *in vitro*, might be blurred in human cells by the concomitant presence of murine C-type virus antigens.

Summary. The synthesis of murine tumor virus group-specific (GS) and virus envelope (V) antigens following infection of mouse, rat, hamster, chicken, and human cells with mouse sarcoma virus, strain moloney (M-MSV), was studied by immunofluorescence (IF). The two antigens were detected in *mouse* embryo cells as early as 12–15 hr post-infection (pi), reaching maximal levels at 24–48 hr. An early cytopathic effect (CPE) involving the whole monolayer was constantly present at 24 hr although a fraction only of the cells were actively synthesizing viral antigens. Identical results, except that cellular alteration was absent, were obtained after infection of *rat* embryo cells; whereas *chicken* and *hamster* cells remained totally negative. Infection of *human* diploid cell lines resulted in the appearance of murine GS and V antigens and CPE 10 days pi. The proportion of antigen-containing cells was observed to decrease in consecutive subcultures.

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