Immunofluorescent Studies of RD-114 Virus Replication in Cell Culture

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SUMMARY

Newly formed gs-I antigen of RD-114 virus was detected by the indirect fluorescent antibody (IFA) procedure in the cytoplasm of the D-17 dog cell line at 24 h post-infection in approx. 30% of the cells. Virus-induced membrane antigen was present in approx. 50% of the cells by 48 h, at which time new infectious virus was being produced. Polykaryons were observed in infected cells. Their formation paralleled production of virus, as determined by the IFA membrane staining technique.

INTRODUCTION

The group-specific (gs) antigen of avian leukaemia viruses (ALV) was first identified by the complement fixation technique (Huebner et al. 1964); later, other workers (Kelloff & Vogt, 1966; Payne, Solomon & Purchase, 1966) used the immunofluorescence technique to study the gs antigen of ALV in infected cells. Other reports described the use of immunofluorescence technique to study the gs antigen of murine (Lejneva & Abelev, 1970; Chuat et al. 1971) and feline (Ubertini et al. 1971) leukaemia viruses. Hampar and co-workers (Hampar et al. 1971) also used this technique to study the gs-I antigen of both murine and hamster type-C viruses using antisera to gs-I antigen which had been purified by electrofocusing (Gregoriades & Old, 1969).

The present report describes the use of immunofluorescence to detect the presence of the gs-I antigen of the RD-114 virus within infected cells (McAllister et al. 1972; Oroszlan et al. 1972) and to attempt correlation of the temporal relationship of the formation of the antigen in these cells with virus-induced cell surface antigens and with newly formed infectious virus.

METHODS

Virus. The virus used was the RD-114 virus propagated in the RD-114 cell line. Samples of virus for use in this study were obtained from the chronically infected monolayer cultures which had been fed 3 days previously. These virus samples were used immediately without freezing or storage and when assayed for infectious virus content by induction of specific virus-induced membrane antigen (see below), consistently showed titres of 10^6 infectious units per ml.

Cell culture. The cell line used in this study was a dog cell line designated as D-17 and established in this laboratory from a metastasis to the lung of an osteosarcoma in a standard poodle dog. This line has been extensively monitored by electron microscopy for type-C
particles and none have ever been observed. The cells used were in passage 190 to 200 and were grown in Eagle’s minimal essential medium supplemented with 10% foetal bovine serum and containing penicillin, streptomycin, and fungizone.

**Complement fixation tests.** The complement fixation reaction used for the detection of antigen and antibody was the standard test adapted to the microtitre technique in our diagnostic laboratory (Lennette, 1969).

**Purification of gs-1 antigen.** The gs-1 antigen was purified by electrofocusing, essentially by the method described by Oroszlan et al. (1970). Concentrated RD-114 virus (10¹¹ particles per ml) was provided by the Office of Program Resources and Logistics of the Virus Cancer Program through the courtesy of Dr J. Gruber. A 3 ml sample of the concentrated virus was combined with 16 ml of saline and 1 ml of a solution of 2% Tween 80 and the mixture was stirred mechanically for 1 h at room temperature. An equal vol. of ether was then added and stirring continued for an additional hour. The aqueous phase was recovered by sedimentation and two additional ether extractions were carried out. After the final extraction, residual ether was removed from the aqueous phase by a stream of nitrogen gas. The aqueous phase, which constituted approx. 15 ml, was then centrifuged at 100,000 g for 1 h and the supernatant fluid applied to the electrofocusing apparatus with an ampholyte gradient of pH 3 to 10 stabilized with a sucrose gradient of 0 to 40%. Electrofocusing was carried out at 300 V for 72 h. Thirty drop fractions were then collected; these were tested for their pH and $E_{280}$, for complement fixing activity using guinea pig antiserum specific for RD-114 gs-1 antigen kindly supplied by Dr Ray Gilden, Flow Laboratories, Rockville, Md.

**Antiserum to purified gs-1 antigen.** Antiserum to the purified gs-1 antigen was prepared in guinea pigs. The fractions from the electrofocusing column showing antigenic activity by the complement fixation reaction of $>1:16$ were combined and utilized as antigen. The first injection consisted of equal vol. of antigen and complete Freund’s adjuvant inoculated into the footpads of the guinea pigs. Five additional injections of antigen without Freund’s adjuvant (0.5 ml) were made at weekly intervals, a portion intramuscularly and the remainder subcutaneously. Ten days following the final injection, the animals were bled and the sera assayed for complement fixing activity. Those animals with complement fixing titres of $>1:32$ against concentrated ether-treated RD-114 virus were exsanguinated and the sera were stored frozen at $-70^\circ C$ until use. The antisera, when tested by immunodiffusion against concentrated ether-treated RD-114 virus and concentrated ether-treated feline leukaemia virus (FeLV), reacted with a single line against only the RD-114 virus preparation.

**Antiserum to whole virus.** Antiserum to sucrose banded RD-114 virus was prepared by injecting two rabbits with approx. 750 µg of virus protein and complete Freund’s adjuvant. Six additional intraperitoneal injections of purified virus (140 to 750 µg virus protein per injection) without Freund’s adjuvant were administered periodically (21 to 333 days after the first) and serum collected 10 days after each injection. The sera used in these studies contained neutralizing antibodies for RD-114 virus (Klement & McAllister, 1972) and antibodies to RD-114 virus-induced cell surface antigens but not feline leukaemia virus-induced cell surface antigens (Boone, Church & McAllister, 1973).

**Anti-species antibody.** Goat anti-guinea pig γ-globulin and goat anti-rabbit γ-globulin were purchased from Antibodies Inc., Davis, California, and were labelled with fluorescein isothiocyanate. Following labelling, the conjugates were tested by immunoelectrophoresis for their ability to react with the γ-globulin in the serum from the species of animal to which the antibodies were directed.
Immunofluorescence test. The indirect fluorescent antibody (IFA) procedure was used in this study. Before use in the staining procedure, the intermediate sera were absorbed twice with foetal bovine serum which had been insolubilized by the procedure of Avrameas & Ternynck (1969) and twice with uninfected D-17 cells (0·1 ml of packed cells per ml of serum).

For assaying gs-I antigen, the cells on coverslips were washed, fixed in acetone for 5 min, air dried and reacted with a 1:5 dilution of the guinea pig anti gs-I antigen either at 37 °C for 1 h or overnight at 4 °C. After rinsing with phosphate-buffered saline (PBS), pH 7·2, the fluorescein labelled anti-species (guinea pig) antibody was reacted with the cells on the coverslip for 1 h at 37 °C. Following washing with PBS, the coverslips were mounted in Elvanol mounting medium and examined with the fluorescent microscope fitted with the Zeiss FITC interference filter. Illumination was provided by a 12 V 100 W halogen lamp. Black and white photographs were made, however, utilizing the usual HBO 200 Osram burner as a light source with UG-1 exciter filter. Virus-induced membrane antigen was detected by using the rabbit anti virus antibody at a 1:5 dilution as the intermediate serum in the IFA procedure as was described previously (Riggs, 1971).

Controls for both the gs-I antigen staining and staining for virus-induced membrane antigen consisted of the following: (a) utilizing uninfected D-17 cells in the staining procedures; (b) using D-17 cells infected with feline leukaemia virus in the staining procedures; and (c) using normal rabbit or normal guinea pig sera as the intermediate reactant in the staining procedures with the RD-114 virus infected D-17 cells. All controls were consistently negative.

Detection of gs-I antigen. Freshly harvested D-17 cells were infected in suspension with RD-114 virus (input multiplicity of about 1 infectious unit per cell). Adsorption was allowed to proceed for 3 h, the cells were washed twice with growth medium, plated in 50 mm plastic Petri dishes containing coverslips, and incubated at 36 °C in a humidified atmosphere of 5% CO₂ in air. Coverslips were removed at daily intervals after plating, and were fixed and stained by the IFA procedure for detection of gs-I antigen.

Virus-induced membrane antigen. D-17 cells which were infected with RD-114 virus as described above were plated in 8 oz prescription bottles (10⁶ cells per bottle). At 24 h intervals after infection, samples of supernatant medium were removed from the bottles for virus assay while the cells were utilized in the IFA procedure for virus-induced membrane antigen. Culture fluids were not changed during this experimental procedure. The supernatant fluid was filtered through a 0·45 μm millipore filter; tenfold dilutions of the filtrate were then made and 1 ml portions were added to freshly harvested D-17 cells in suspension as described above. The cells were then plated in 8 oz prescription bottles and were subcultured at weekly intervals (1:20 split) and one feeding between subcultures. After 3 weeks in culture, the cells were used in the IFA procedure to detect virus-induced membrane antigen.

RESULTS

gs-I antigen purification

When the fractions from the electrofocusing column were screened for gs-I antigen, the fractions showing activity appeared in the gradient at pH 8·5 to 8·7. Fig. 1 shows the results of one of the experiments from the electrofocusing procedure. Only the fractions from pH 6 to 10 are depicted since all of the gs-I antigen activity was consistently found within this pH range.
Fig. 1. Isoelectric focusing of Tween-ether extracted RD-114 virus. Fractions 10 to 35 are shown (pH 6 to 10). The ampholyte gradient was from pH 3 to 10 stabilized with a sucrose gradient of 0 to 40%. Electrofocusing was carried out at 300 V for 72 h. •—•, E_{260}; ○—○, complement fixation (reciprocal of dilution); ▲—▲, pH.

Fig. 2. Growth of RD-114 virus in D-17 cells. Samples of supernatant fluid of infected cells were removed at the indicated time intervals and were assayed for infectious virus by the virus induced membrane staining technique (see text).

Detection of virus-induced membrane antigen

The production of new infectious virus following infection of the D-17 cells with RD-114 virus, as determined by virus-induced membrane antigen, is shown in Fig. 2. These results were obtained when the supernatant fluids were assayed for infectious virus for six daily intervals following infection. At 24 h the titre of virus had fallen to 10^3 infectious units.
Immunofluorescence of RD-114 virus

Fig. 3. D-17 cells infected with RD-114 virus and stained by the immunofluorescence technique for gs-1 antigen at various times after infection. (a) Uninfected control. (b) 24 h post-infection. (c) 2 days post-infection. (d) 3 days post-infection. (e) 4 days post-infection. (f) 6 days post-infection. Magnification x 675.

per ml while at 2 days following infection the titre had risen to 10^5. During the following time periods, 10^8 infectious units were recovered, probably reflecting some inactivation of viable virus with further incubation without feeding the cultures.

Detection of gs-1 antigen

Newly synthesized gs-1 antigen could be detected at 24 h post-infection. Fig. 3 shows the results at various time intervals after infection. At day 1 the antigen appeared in the cytoplasm, always at a juxtanuclear position with the nucleus remaining negative. The number of cells showing such staining at this time period was approx. 20%. At day 2 post-
Table 1. Comparison of staining reactions of D-17 cells infected with RD-114 virus and stained by IFA for membrane antigen or gs-I antigen

<table>
<thead>
<tr>
<th>Time post-infection (days)</th>
<th>Virus membrane antigen</th>
<th>gs-I antigen</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0*</td>
</tr>
<tr>
<td>1</td>
<td>30%+</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>60%+</td>
<td>50%+</td>
</tr>
<tr>
<td>3</td>
<td>Polykaryon formation†</td>
<td>≥90%+</td>
</tr>
<tr>
<td>4</td>
<td>Polykaryon formation</td>
<td>≥90%+</td>
</tr>
</tbody>
</table>

* Majority of cells showed a very dull punctate staining pattern probably due to adsorbed antigen.
† Multinucleated cells all stained. Further counts could not be made for single cells.

infection, the staining extended further into the cytoplasm and a very dull staining was evident in the nucleus. It was difficult, however, to determine if the antigen was actually within the nuclear membrane or whether the staining was due to cytoplasmic antigen superimposed over the nucleus. Approx. 60 % of the cells showed gs-I antigen production at this time. By day 3 post-infection extensive staining throughout the cells was evident. Counts of infected cells could not be made because of multinucleated cell formation by this time period. All of the multinucleated cells stained to some extent; therefore, the percentage of cells showing gs-I antigen from this time on could not be determined. At day 4 and 6 post-infection the staining increased in intensity and the majority of the cells stained.

A comparison of the number of cells staining at different time periods after infection by membrane staining and gs-I antigen staining is made in Table 1. As can be seen, gs-I staining was evident in 30 % of the cells at 1 day post-infection while membrane staining was not evident until 2 days post-infection.
Immunofluorescence of RD-114 virus

Polykaryon formation

Normally the D-17 cell monolayer consists of cells showing only an occasional giant cell or polykaryon (Fig. 4a). Following infection with the RD-114 virus, however, extensive polykaryon formation was noted, as is shown in Fig. 4b. This type of c.p.e. which consists of giant cells and cells with from 2 to 15 nuclei, continued with subculturing of the infected cells until approx. the fourth passage, when the cell line assumed its normal appearance. The cells, however, continued to produce virus and were considered chronically infected cultures.

The timing of polykaryon formation corresponded exactly to the production of infectious virus. When the dilutions of virus from the time study were inoculated onto the D-17 cells, the cultures showing extensive polykaryon formation stained for virus-induced membrane antigen in the IFA procedure. Those from the dilutions past the end-point of infectious virus showed no excessive polykaryon formation and did not stain by the IFA procedure.

DISCUSSION

The newly formed gs-1 antigen appeared in the cytoplasm of the D-17 cell by 24 h following infection. Other workers have reported murine gs antigen visible as early as 15 h post-infection (Chuat et al. 1971). In our system the nucleus at this time showed no staining whatsoever; therefore, it seems that if the nucleus takes part in the early gs-1 antigen synthesis, the concentration of the antigen in this structure is below the level of the sensitivity of the IFA test used. By 48 h the antigen was being produced in approx. 60% of the cells and at this time period new infectious virus was first detected. Some staining for gs-1 antigen was evident at this time either in or over the nucleus, although at no time period was the intensity of staining equal to that in the cytoplasm. The predominance of cytoplasmic staining would be in accord with the results reported by other workers with different virus-cell systems (Kelloff & Vogt, 1966; Lejneva & Abelev, 1970; Chuat et al. 1971; Hampar et al. 1971; Ubertini et al. 1971).

The drop in titre of infectious virus at later time periods probably reflects some inactivation of viable virus with further incubation. Normally the growth medium is replaced on the cultures on a 3- to 4-day schedule and once the D-17 cells are chronically infected, the fluids consistently show an infectious virus titre of $10^5$ to $10^6$ infectious units per ml.

The polykaryon formation in the newly infected D-17 cells followed exactly the titre of infectious virus, as determined by the IFA membrane staining technique. The polykaryons or giant cells were present in the infected cultures until approximately the fourth subculture, at which time the chronically infected cultures reverted to the normal appearance of the D-17 cell. These findings suggest a mixture of cell types in the D-17 cell line, some susceptible to infection with a subsequent giant cell or polykaryon-formation type of c.p.e. Apparently these types of cells will not survive subculturing. Cloning experiments with the D-17 cell line should clarify this question and we are presently determining which of the known mammalian type-C viruses produce this type of c.p.e. in the D-17 cell line. An alternative explanation for the loss of polykaryons with subculturing could be that when all of the cells become infected, polykaryon formation is no longer possible. With an input multiplicity of approx. one infectious unit per cell, all of the cells would not become infected, possibly allowing polykaryon formation until sufficient progeny virus infected every cell. An analogous situation was found by Clarke et al. (1970), who produced carrier cultures of hamster and human cell lines infected with the type 1 simian foamy agent.
RD-114 virus has induced syncytial c.p.e. or polykaryon formation in human cancer cell lines (Klement & McAllister, 1972; Rand & Long, 1972) and in human lymphoblastoid cell lines (Hampar et al. 1973); in the latter, as in the D-17 cells, the polykaryons gradually disappeared from the infected cultures. Although the identity of RD-114 virus has been established (Livingston & Todaro, 1973), the studies reported here confirm earlier reports that the envelope and gs-I antigens of the virus (McAllister et al. 1972; Oroszlan et al. 1972) and the cell surface antigens induced by the virus are different from those of the previously reported feline type-C viruses.

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REFERENCES


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