Isolation of Helper Viruses from Preparations of Hamster-specific Sarcoma Viruses

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SUMMARY

Non-transforming helper viruses were isolated from pools of two hamster-specific sarcoma viruses in which isolations by terminal dilution procedures had previously been unsuccessful. This presumably resulted from approximately equivalent levels of sarcoma and helper viruses in the original pools. Helper virus was isolated from morphologically normal cells selected from tissue culture plates showing relatively few transformed colonies, and by end-point dilution from cloned transformed cells. Focus-formation by the hamster-specific sarcoma viruses was independent of the spread of virus and non-producer cell lines carrying the sarcoma virus genome were isolated; thus these viruses were apparently unable to replicate independently of helper virus. Hamster-specific virus antigens were not detected in hamster cells infected productively or non-productively with a murine sarcoma virus; thus, evidence for virus activation in vitro by the sarcoma virus genome was not obtained.

INTRODUCTION

In previous reports, we described immunological relationships among three hamster-specific sarcoma viruses isolated from hamster tumours induced by murine sarcoma viruses (Kelloff et al. 1970a, b, c). These three hamster sarcoma viruses were shown to share at least one envelope antigen and an internal ether-resistant antigen with a hamster helper virus (HaLV) isolated from preparations of one of these viruses, M-MSV(HaLV)G. Further, an indigenous hamster C-type virus (Stenback, Van Hoosier & Trentin, 1968) was also shown to share this internal ether resistant (group-specific – gs) antigen (Kelloff et al. 1970c). This led us to postulate that the three hamster sarcoma viruses were pseudotypes having their host-range and antigenic specificity supplied by this indigenous hamster virus or a similar agent.

This report establishes the presence of a helper virus in the two other hamster sarcoma virus preparations, Ki-MSV(HaLV) and M-MSV(HaLV) and establishes the presence of defective sarcoma viruses in the three sarcoma virus stocks. We also describe unsuccessful attempts to detect hamster C-type virus activity in hamster embryo fibroblasts infected singly with a defective murine sarcoma virus.
METHODS

**Viruses.** The sources and nomenclature of the three hamster sarcoma viruses: m-MSV(HaLV)G, m-MSV(HaLV) and ki-MSV(HaLV), as well as the hamster helper virus, HaLV, have been described (Kelloff et al. 1970a,b). Virus pools for these studies were made from tumour transplant lines (Moloney, 1960) or from virus-infected tissue cultures. The Rauscher pseudotype of Moloney sarcoma virus, m-MSV(RLV), adapted to growth in rat cells was a generous gift of Dr R. C. Ting (Ting & Bader, 1969).

**Tissue culture.** Tissue-culture methods, focus assays, and interference assays have been described (Kelloff et al. 1970b). Rescue experiments were performed according to published methods (Kelloff et al. 1970b; Huebner et al. 1966). For this purpose \(3 \times 10^5\) HT-1 cells (Huebner et al. 1966) were co-cultivated with \(3 \times 10^5\) cells from clones which were thought to contain HaLV. Conversely, cell lines known to shed HaLV (Kelloff et al. 1970b) or RLV (Chang et al. 1969) were co-cultivated with cell lines which were thought to contain the defective MSV genome. At 7 and 14 days, virus harvests were made and tested for focus-forming activity on LSH hamster embryo fibroblasts.

**Antisera.** Neutralizing antisera were obtained from weanling hamsters bearing transplants of a tumour line originally induced by m-MSV(HaLV)G, and from guinea-pigs hyperimmunized with m-MSV(HaLV)G purified by rate-zonal centrifugation in sucrose density gradients. An antiserum pool was obtained which completely neutralized 80 focus-forming units of m-MSV(HaLV)G at a dilution of 1/160. This antiserum pool was used at a final dilution of 1/40 in the cloning experiments. The preparation of antisera to the group-specific antigen of the hamster C-type viruses has been described (Kelloff et al. 1970c; Oroszlan et al. 1971). This antiserum was used in complement-fixation tests (Huebner et al. 1963) to determine the presence of hamster C-type virus antigens in various preparations. Cell-pack antigens (20 %, v:v) were treated with Tween 80 + ether before testing.

**Cloning techniques.** Hamster embryo fibroblasts were inoculated with dilutions of hamster sarcoma viruses. Neutralizing antiserum was added to the medium 24 hr after infection and was also present in fresh medium added 3 days later. Foci of transformed cells were recognized readily 6 days after infection. At this time, the foci were transferred with a 2 mm. cloning ring to 60 mm. Petri dishes. These cells were grown in Eagle's minimal essential medium with 10 % foetal calf serum, and colonies appearing to arise from single cells were recloned by the same methods. Areas of morphologically normal cells in dishes containing only a few transformed foci were cloned by the same methods in attempts to isolate helper viruses potentially present in the sarcoma virus pools.

**[3H]-uridine labelling of virus particles.** Various tissue culture preparations were tested for virus synthesis by following the incorporation of \([3H]\)-uridine into particles having a buoyant density of 1.16 g./ml. in sucrose gradients (Duesberg & Robinson, 1966).

**Electron microscopy.** These techniques have been described (Kelloff et al. 1970a). A culture was considered positive for virus if typical budding or extracellular C-type particles were seen clearly.

RESULTS

**Kinetics of focus-formation**

Previous studies of m-MSV(HaLV)G established that focus formation follows one-hit kinetics and suggested that HaLV, known to be present in only tenfold excess of the sarcoma virus, is not required for focus formation (Kelloff et al. 1970b). The likely alternatives are that focus formation results from a defective sarcoma virus, not depending on virus
replication, or from a competent sarcoma virus. Cloning experiments were necessary to distinguish these alternatives and, since cloning experiments were also planned for m-MSV(HaLV) and ki-MSV(HaLV), the kinetics of focus-formation by these viruses were examined.

Hamster embryo fibroblasts were infected with dilutions of the three sarcoma viruses. To prevent virus spread, neutralizing antiserum was added to the medium 24 and 96 hr after infection. Foci were counted on day 7 and were consistent with a one-hit titration pattern. Calculated infectivities (focus count x dilution reciprocal) showed an increase at higher dilutions of from 18 to 25% greater than expected for a one-hit pattern; this was probably due to interference and infectivities were thus clearly distinct from those expected for a two-hit curve. Supernatant fluids from plates containing 80 to 90 foci were assayed on day 7 and no infectivities greater than three f.f.u./ml. were obtained from undiluted fluids, attesting to the effectiveness of the neutralizing antisera. Control plates containing no antisera yielded as many as 2.5 x 10³ f.f.u./ml. This one-hit pattern is similar to that found for m-MSV (HaLV)G (Kelloff et al. 1970b).

Isolation of non-producer foci

In previous reports (Kelloff et al. 1970a,b), it was established that m-MSV(HaLV)G contained a helper virus, HaLV, present at a level tenfold above that of the focus-forming virus. No helper virus was found by end-point dilution tests in m-MSV(HaLV) and ki-MSV(HaLV) preparations. It was assumed therefore that plates receiving a low dose of sarcoma virus (about 5 to 10 f.f.u.) and containing neutralizing antibody in the medium as above, would yield some foci of transformed cells that were free of helper virus. Plates were infected with 5 to 10 f.f.u. of each of the three hamster sarcoma viruses, m-MSV(HaLV)G, m-MSV(HaLV) and ki-MSV(HaLV). Foci of transformed cells were transferred to 60 mm. Petri dishes and as colonies of transformed cells grew up from single cells, they were recloned on new dishes. Normal cells were present in the initial colonies, but these were easily distinguishable from the transformed cells. As early as possible the clones were screened in complement-fixation tests with antisera prepared against the hamster C-type virus group-specific antigen. Four clones derived from m-MSV(HaLV)G (one), m-MSV(HaLV) (two), and ki-MSV(HaLV) (one) were found to be non-reactive in complement-fixation tests.

<p>| Table 1. Properties of transformed cloned cell lines induced by three hamster-specific sarcoma viruses |
|---------------------------------|-------|---------------|-------------|----------|</p>
<table>
<thead>
<tr>
<th>Inducing virus</th>
<th>Number obtained</th>
<th>Focus forming virus</th>
<th>Physical particles</th>
<th>Helper virus</th>
<th>MSV genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clones negative for HaLV gs antigen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-MSV(HaLV)G</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>m-MSV(HaLV)</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>ki-MSV(HaLV)</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Clones positive for HaLV gs antigen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-MSV(HaLV)G</td>
<td>6</td>
<td>+</td>
<td>NT</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>m-MSV(HaLV)</td>
<td>11</td>
<td>+</td>
<td>NT</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>ki-MSV(HaLV)</td>
<td>3</td>
<td>+</td>
<td>NT</td>
<td>+</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Direct assay of frozen-thawed cells on hamster embryo fibroblasts.
† [3H]-uridine labelling and electron microscopy of thin sections.
‡ Non-focus forming virus beyond focus end-point capable of rescuing MSV genome from HT-1 cells.
§ Rescue of focus forming virus after co-cultivation with HaLV or RLV shedding cell lines.
NT = not tested.
Subsequently, no virus was detected by electron microscopic examination, by the [3H]-uridine labelling technique, or by direct isolation attempts. However, these cells contained the sarcoma virus genome since upon co-cultivation with a continuous hamster line shedding HaLV (Kelloff et al. 1970b), or a continuous mouse line shedding RLV (Chang et al. 1969), focus-forming viruses were obtained which were active on hamster and mouse embryo fibroblasts, respectively (Table 1).

Clones reactive in complement-fixation tests with hamster anti-gs antisera were all shown to contain focus-forming virus when assayed on hamster embryo fibroblasts. Assay plates receiving inocula at a tenfold dilution higher than the focus-forming end-point were found to be consistently reactive in complement-fixation with anti-hamster gs serum. Following our experience in isolation of HaLV (Kelloff et al. 1970a,b), this is presumptive evidence that all of these 'producer' clones contain helper virus; thus, there is no direct evidence that a competent sarcoma virus is present in these clones. These results are summarized in Table 1.

**Presence of helper virus in the preparations of M-MSV(HaLV and Ki-MSV(HaLV)**

Preparations of M-MSV(HaLV) and Ki-MSV(HaLV) have not been shown to contain helper virus by end-point dilution techniques (Kelloff et al. 1970a); cloning experiments were therefore done to examine these virus pools for helper virus. Twenty clones of normal cells were obtained from plates infected with 5 to 10 f.f.u. of the two sarcoma viruses. After one re-cloning, these clones were passaged and screened in the complement-fixation test with guinea-pig anti-hamster gs antisera. Two clones derived from M-MSV(HaLV) infected cells and two clones derived from Ki-MSV(HaLV) infected cells were found to be positive. Electron microscopic examination of these four lines revealed C-type particles. Cell lines derived from these clones yielded no focus-forming virus, but when co-cultivated with HT-1 cells, the harvests yielded focus-forming virus on hamster embryo fibroblasts (Table 2). We assume that helper virus was present in the stocks of these sarcoma viruses, but was not necessarily present in excess of the latter.

**Attempts to detect the indigenous hamster C-type virus in hamster embryo fibroblasts**

Since *in vivo* rescue of HaLV was shown with all three input murine sarcoma viruses, attempts were made to achieve this *in vitro*. A murine sarcoma virus, M-MSV(RLV), capable of growth on hamster cells was kindly supplied by Dr R. C. Ting. This virus contained only murine C-type virus antigens so that the presence of hamster virus antigens in infected hamster cells could be assumed to originate in these cells.

Tissue culture plates of hamster embryo fibroblasts were infected with 5 to 10 f.f.u. of

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**Table 2. Isolation of helper virus from morphologically normal cells in cultures infected with sarcoma viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Proportion CF positive</th>
<th>Presence of virus in CF positive cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Physical particles</td>
</tr>
<tr>
<td>M-MSV(HaLV)</td>
<td>2/20</td>
<td>2/2</td>
</tr>
<tr>
<td>Ki-MSV(HaLV)</td>
<td>2/23</td>
<td>2/2</td>
</tr>
</tbody>
</table>

* Cell lines which were negative in the complement-fixation test (CF negative) were tested by [3H]-uridine labelling and microscopy and showed no physical particles. Tests were also negative for focus-forming activity, and ability to rescue the MSV genome from HT-1 cells by co-cultivation.
m-MSV(RLV). On day 6, foci were cloned from several plates and recloned as described above. The murine gs antigen was detected by complement-fixation in seven of eight clones tested. The one negative clone was shown in rescue experiments to contain the defective MSV genome. This clone, and the murine gs antigen positive clones, did not react with hamster gs antisera. Thus the hamster C-type virus information which was potentially present in these hamster embryo fibroblasts was not ‘activated’ by infection of the cells with replicating or defective murine sarcoma virus.

**DISCUSSION**

Previous studies have shown the presence of a helper virus, HaLV, in m-MSV(HaLV)G preparations and have also shown the immunological identity of the group-specific antigen of HaLV with those of the three hamster sarcoma viruses and the agent from D-9 (Kelloff et al. 1970a, b). This suggested that the three hamster sarcoma viruses consisted of the sarcoma genomes of the original tumour-inducing murine viruses, with antigenic and host-range specificities supplied by structural proteins coded for by an indigenous hamster helper virus. The finding of helper viruses in the stocks of ki-MSV(HaLV) and m-MSV(HaLV) provided further evidence for this premiss. In addition, isolation of non-producer transformed cells from cultures inoculated with each of the three sarcoma viruses provided direct evidence that these viruses were unable to replicate independently of helper virus. Although a high percentage of non-producer foci was not obtained, all the producer foci contained non-transforming virus in excess of the focus-forming virus, suggesting the presence of a helper virus. Conversely, no evidence for the presence of a competent hamster-tropic sarcoma virus was found. The inability of MSV to replicate independently of helper virus has been reported (Huebner et al. 1966; Aaronson & Rowe, 1970).

It appears that indigenous hamster C-type viruses can be obtained regularly from hamster tumours induced by murine sarcoma viruses. This phenomenon is somewhat unexpected since, with few exceptions (Stenback et al. 1968; G. Kelloff, R. J. Huebner, W. T. Lane & R. V. Gilden, unpublished results), C-type viruses have not been detected by the conventional screening methods of complement-fixation and electron microscopy. Similarly, C-type viruses have not been detected in attempts to isolate them directly from various organs and tumours from uninoculated hamsters and hamsters bearing chemically induced or DNA virus-induced tumours. Infectious virus probably exists in these animals but in quantities too small to be detected by these methods. The reason for the detection of infectious HaLV in MSV-induced tumours is not known but suggests ‘activation’ of a latent virus. The failure to detect any HaLV activity in vitro in hamster embryo fibroblasts infected singly with a defective sarcoma virus suggests that ‘activation’ of HaLV may require other conditions not present in vitro. One possibility is that HaLV is a passenger from another source, such as the haematopoietic tissues.

Cell fusion techniques are now being used to maximize the possibility of rescue.

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REFERENCES


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