Proteins of the Murine C-Type RNA Tumour Viruses: Isolation of a Group-specific Antigen by Isoelectric Focusing

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SUMMARY

The group-specific antigen of two murine C-type RNA tumour virus types was purified to homogeneity by isoelectric-focusing of virus disrupted with tween-ether. The antigen has a molecular weight of 25,000 to 26,000 (estimated from sedimentation in sucrose relative to standards) and an isoelectric point of 6·7. The latter value was obtained from preparations of Friend, Moloney, Rauscher and Gross subgroups. The antigen was immunogenic in guinea-pigs inducing the synthesis of monospecific group-reactive antibodies. The antigen was relatively free of nucleic acid and appeared to be the only virus protein, based on labelling studies, with an isoelectric point near neutrality. Envelope antigen was localized at pH 4·5 by the electrofocusing technique.

INTRODUCTION

The murine and avian C-type RNA tumour viruses are characterized by a species-specific group-reactive internal virion antigen (gs antigen) (Huebner et al. 1964; Huebner, 1967). Antisera reactive with this antigen have been essential reagents for tissue culture assays of the non-cytopathogenic C-type viruses, i.e. the complement-fixation test for avian leukaemia viruses (COFAI test) (Sarma, Turner & Huebner, 1964) and the complement-fixation test for murine leukaemia viruses (COMUL test) (Huebner, 1967; Hartley et al. 1969). In general, the appropriate antisera have been most readily obtained from tumour-bearing animals of heterologous species (Hartley et al. 1969; Huebner et al. 1964), although immunization with virus preparations, with absorptions where necessary, has also yielded group-reactive antisera (Geering, Old & Boyse, 1966; Bauer & Schäfer, 1966; Schäfer & Seifert, 1968). Immunization procedures might be expected to be of greater importance for producing antisera to other mammalian C-type viruses, e.g. human when obtained, and therefore thorough study of the murine and other model systems in this regard is well warranted. Estimations of the number and properties of the gs antigen(s) have been somewhat conflicting, partially because of the heterogeneous nature of the antigen under certain conditions of extraction (Gregoriades & Old, 1969). This report describes procedures leading to the purification of the murine gs antigen to a monodisperse, immunogenic form.

METHODS

Virus. AKR virus was obtained from continuous cultures of a virus-producing rat lymphosarcoma initiated with this mouse leukaemia virus strain. Concentrated Rauscher mouse plasma virus was obtained from the Program Resources and Logistics Section, Viral
Oncology Program, National Cancer Institute. Rauscher leukaemia virus was also obtained from a continuous murine cell line, F-4, which actively produces this virus.

Cell culture. The continuous AKR rat lymphosarcoma cell line was grown in C-32 oz. bottles or in roller bottles (Type R Dt. 685; 6·4 × 68·5 cm. surface area 1200 cm.², Flow Laboratories, Inc., Rockville, Maryland) in Eagle's basal medium supplemented with 10% foetal bovine serum and containing 2 % (w/v) glutamine, 100 units/ml. each of penicillin and streptomycin and 0·02 % neomycin sulphate. Cells were fed with fresh medium every 4 days and sub-passaged every 8 days.

Radioactive virus. To obtain [3H]uridine labelled virus, monolayers of AKR rat lymphosarcoma cells in roller bottles, when 70 to 80% confluent, were incubated at 37° with 50 ml. fresh medium containing 20 µCi/ml. [3H]uridine (20 c/mmole) for 24 hr. The medium was then collected and replaced with 50 ml. fresh medium for another 24 hr. The fluid harvests were pooled and the virus concentrated and purified as described below. For labelling of virus with [3H]leucine, leucine free medium containing 1/10 of the normal concentration of other amino acids, 2 % calf serum and 5 µCi/ml. of [3H]leucine (40 c/mmole) was used.

When [14C]amino acid mixtures (0·5 µCi/ml.) were used to label virus, non-radioactive amino acids were not included in the medium.

Purification of virus. Virus-containing tissue culture fluids were harvested every 4 days. After removal of cell fragments and whole cells by low speed centrifugation, the virus was pelleted at 35,000 g for 2 hr and then resuspended in 0·05 M-tris buffer (pH 7·2), containing 5·0 % sucrose. Both tissue culture and plasma virus preparation were purified by centrifugation in 20 to 60 % (w/v) sucrose gradients prepared in 0·05 M-tris+HCl buffer (pH 7·2) with or without 0·001 M-EDTA and 0·1 M-NaCl. Centrifugation was performed in the Spinco SW 41 rotor at 40,000 rev./min. for 90 min. or in the SW 25·1 rotor at 25,000 rev./min. for 3 hr.

Disruption of virus. Virus purified by density gradient centrifugation was disrupted by Tween-ether treatment. The concentration of Tween 80 in the different virus preparations ranged from 0·1 to 0·2 %. Two somewhat different procedures were used.

(a) Virus suspensions containing detergent were vigorously mixed at room temperature with 4 to 5 volumes of washed anaesthetic grade ether for 5 min. Nitrogen gas was then vigorously bubbled through the mixture until the ether completely evaporated. This procedure is similar to that used by Geering et al. (1966). The time necessary to complete the treatment was 0·1 to 1 hr depending on the original volume of the virus preparation.

(b) Virus suspensions treated with detergent were exhaustively extracted with ether as described by O'Connor et al. (1966), except that extraction was at room temperature. After separation of the phases by centrifugation, residual ether was removed from the water phase with nitrogen gas.

Tween-ether treated virus subunit preparations were clarified at 15,000 rev./min. for 5 min. or were centrifuged at 100,000 g for 40 to 60 min. to remove completely undisrupted virus.

Isoelectric focusing. Isoelectric focusing (Swensson, 1962) was carried out in a 110 ml. capacity column (LKB, Uppsala, Sweden) with 1·0 % carrier ampholyte solution in the pH range of 3 to 10 or 5 to 8 for various periods of time. A sucrose density gradient of 0 to 40 % (w/v) was used to prevent convection. The electrodes were protected with dilute phosphoric acid at the anode and with ethylenediamine at the cathode. With the wider pH gradient (pH 3·0 to 10·0) the potential was 300 v and equilibrium was reached within 24 hr. Experiments in the pH range of 5 to 8 were conducted for 36 to 40 hr with an average potential of 700 to 800 v. The temperature was maintained at 5° with a Lauda K-2/R constant temperature circulator. After completion of the separation, fractions of 1·5 to 2 ml. were
collected and assayed for pH (Orion Model 801 pH meter), radioactivity (Beckman LS 250 liquid scintillation system) and antigenic reactivity. Fractions below pH 5.0 were adjusted to neutral pH with sodium bicarbonate before complement fixation and immunodiffusion tests were made.

**Immunodiffusion and electrophoresis.** Double diffusion tests were carried out on microplates in 0.8% agarose gel prepared in 0.01 M-tris + HCl buffer with 0.9% NaCl and merthiolate (1/10,000) as a preservative.

Immunoelectrophoresis was performed in 0.8% agarose gel in 0.05 M-barbital buffer (pH 8.6) in the cold (4°) with a potential difference of 250 V using the LKB 6800 A apparatus for 2 hr.

**Complement fixation.** The microtechnique (Sever, 1962) was employed using 1.8 units of complement and 4 units of antibody or antigen. Specific activity was taken as titre/mg. protein, without adjustments for test volume. Thus, the titre of the 0.025 ml. sample used in the microtitre test is considered to represent the number of complement fixing (CF) units per ml.

**Antisera.** Rat Sera: M-MSV rat serum obtained from rats bearing transplants of sarcoma induced by the MOLONEY strain of murine sarcoma virus. This serum reacts with envelope and gs antigen of all murine C-type viruses in complement fixation tests (Hartley et al. 1969). AKR rat serum obtained from rats bearing AKR leukaemia-virus-induced lymphosarcoma. This serum is highly specific for AKR (Gross type) envelope antigen. Guinea-pig sera: anti-murine leukaemia virus gs guinea-pig, serum prepared against purified (isoelectrically focused) gs antigen from Rauscher virus as described in this paper.

**Protein determination.** The protein content of antigen preparations was determined spectrophotometrically from the extinction at 280 and 260 nm. (Warburg & Christian, 1941) or by the method of Lowry et al. (1951).

**RESULTS**

**Disruption of virus and release of internal antigen**

In the experiments described below Tween-ether treatment was used to release internal antigen from virus purified on a sucrose density gradient. The degree of disruption of the virion could be estimated by measuring the decrease in turbidity at 550 nm. of the ether-free water phase. Tween-ether treatment was regarded as complete when turbidity in the water phase was minimal. After such treatment, the majority of gs antigen activity (based on gel diffusion tests) was released from the virus particle and appeared in the water phase as a soluble component, i.e. not pelleted by centrifugation at 100,000g for 1 hr. As monitored by gel diffusion initially and later by gs specific guinea-pig antisera in CF tests, freshly banded virus was, at most, weakly reactive; however, after freezing and thawing or simply standing at 5°, gs activity could be demonstrated, indicating virus breakdown.

**Sedimentation of doubly labelled virus before and after Tween-ether treatment**

The above findings were corroborated by results of experiments in which doubly labelled intact and disrupted virus was centrifuged in 15 to 60% sucrose gradients (Fig. 1). Intact virus banded at a density of 1.156 g./cm.³. After complete disruption, all of the H and most of the ¹⁴C activity was recovered in the top fractions of the gradient. Group-specific antigen was found only in these top fractions by both CF and gel diffusion assays, indicating that after Tween-ether treatment this antigen was not present as a large nucleo-protein unit.
The molecular weight of gs antigen was estimated by centrifugation through a 5 to 20 % sucrose gradient (Martin & Ames, 1961). Using myoglobin as a reference standard, the sedimentation coefficient for the CF peak was found to be 2.55 s, from which a molecular weight of 25,000 was calculated in agreement with reported data (Schäfer et al. 1969). In these experiments, reactions with m-MSV rat serum and guinea-pig anti-gs serum were superimposable. Similar values of s were obtained with the gs antigen isolated from both AKR and Rauscher leukaemia viruses.

![Graph](image-url)

Fig. 1. Sucrose density gradient centrifugation of intact and Tween-ether disrupted AKR virus. The virus was labelled with [3H]uridine (● – ●) and [14C]amino acids (■ – ■). Centrifugation was carried out in the Spinco SW 50 rotor at 43,000 rev./min. for 90 min. at 5 ° using 15 to 60 % sucrose gradients. 0.1 ml. of each fraction, collected by bottom puncture, was counted in 10 ml. of toluene base scintillation fluid (Floralloy TLA, Beckman) containing 10 % Bio-Solv (BSS-3, Beckman). (a) Recentrifuged purified virus. (b) The same preparation as in (a) after Tween-ether disruption. Density ▲ – ▲.
Isolation of gs antigen from Rauscher plasma virus

In early experiments designed to isolate gs antigen with electrofocusing techniques, Rauscher plasma virus (10 × plasma concentrates) previously stored at −70 ° was the antigen source. After thawing the virus usually reacted weakly with m-MSV rat serum in agar diffusion, indicating partial disruption and release of gs antigen. The envelope antigen was not detected in these agar diffusion assays. However, similar virus preparations freshly banded on sucrose were negative in these tests even after tenfold concentration. In order to make sure that we were isolating an internal virus antigen, only freshly banded virus was used.

It was disrupted with Tween-ether and the soluble protein components were separated by isoelectric focusing in a pH gradient of 5 to 8 (Fig. 2). The gs antigen, as measured by both CF and immunodiffusion tests with m-MSV rat serum, was localized in a sharp peak at pH 6.7. This activity coincided with a small extinction peak. This was widely separated from most of the u.v. absorbing material which migrated to the acidic side of the pH gradient. Some of the components concentrated in this acidic region appeared to be precipitated but were held in discrete bands in the dense sucrose solution.

Production of specific antisera against electrofocused gs antigen

Group-specific antigen from Rauscher virus was pooled from several electrofocusing experiments and used to immunize guinea-pigs. The antigen was used without further
purification, and without removal of the ampholine and sucrose. The first two injections were given with Freund’s complete adjuvant into the footpad and later injections were given without adjuvant either subcutaneously or into the footpad. The animals received six injections spaced in about 10-day intervals. A total of 70 to 100 CF units (~50 µg. protein) were given to each animal. The yield from about 100 ml. plasma virus was used to immunize three guinea-pigs. The guinea-pigs responded relatively slowly to the antigenic stimulus but did attain CF titres of 1:40 to 1:160.

The antiserum produced appeared highly specific for the gs antigen, reacting only weakly with whole murine leukemia viruses (probably with disrupted virions) and did not react at all with normal cellular antigens (Table 1).

Table 1. Specificity of guinea-pig antiserum prepared against purified Rauscher virus group-specific antigen: comparison with rat antisera of known reactivity

<table>
<thead>
<tr>
<th>Test antigens</th>
<th>m-MSV rat*</th>
<th>AKR rat†</th>
<th>Anti gs-guinea-pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>F, M, R (intact virus)</td>
<td>64§</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>F, M, R (ether disrupted)</td>
<td>64</td>
<td>&lt;4</td>
<td>64</td>
</tr>
<tr>
<td>AKR (intact virus)</td>
<td>64</td>
<td>64</td>
<td>2</td>
</tr>
<tr>
<td>AKR (ether disrupted)</td>
<td>64</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>Purified gs antigen (0.05 mg./ml.)</td>
<td>128</td>
<td>&lt;1</td>
<td>128</td>
</tr>
<tr>
<td>Gel diffusion—all leukaemia viruses—ether disrupted</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* Broad reacting rat antiserum from animals bearing m-MSV transplant tumours. Reactions indicate that this serum reacts with envelope and gs antigens of the major murine C-type virus subgroups.
† Obtained from animals bearing an AKR induced lymphosarcoma. Serum reacts mainly with envelope antigens of AKR virus, no gs reactivity.
§ Reciprocal of CF titre using 4 units of serum.

The antiserum served as a valuable test reagent in further studies in which AKR virus was used.

Isolation of gs antigen from AKR mouse leukaemia virus

Large stocks of virus were obtained from continuous cultures of rat lymphosarcoma cells as described in Methods. With AKR virus most of the electrofocusing experiments were carried out in a pH gradient of 3 to 10. In order to determine more accurately the isoelectric point of the gs antigen of this wild type mouse leukaemia virus, a shallow gradient of pH 5 to 8 was also used.

The results obtained are shown in Fig. 3. Undisrupted virus was localized at pH 4.0 based on reactivity with the envelope specific AKR rat serum. The CF activity was superimposable on an extinction peak at this pH (3a). Disrupted virus yielded a major CF reactive component running at pH 6.7 using both the m-MSV rat antiserum (3b) and the guinea-pig anti-Rauscher gs serum (3c). This component gave a reaction of identity with ether-disrupted Friend, Moloney, Rauscher, Gross and AKR leukaemia viruses in gel diffusion tests, and was also found immunologically identical in similar tests to the gs-1 component isolated by Gregoriades & Old (1969). The latter point was established by an exchange of reagents with these workers. The antigen was homogeneous in polyacrylamide gel electrophoresis (single band) and immunoelectrophoresis (Fig. 4) and recovery of the input gs antigen after electrofocusing in several experiments was virtually 100% (Table 2). [3H]amino acid labelled disrupted virus also showed a major component with an isoelectric point of 6.7, corresponding
Murine C-type virus group-specific antigen 7

To about 20% of the total labelled viral protein (3d). The purified gs antigen had a $E_{280}/E_{600}$ ratio of 1.61 corresponding to a nucleic acid content of about 0.25% (Warburg & Christian, 1941). Disrupted [H]uridine labelled virus, when electrofocused, showed no association of radioactivity with the gs antigen (Fig. 5). Based on these results and the failure of RNase treatment to alter the isoelectric point, the purified antigen is assumed to be free of nucleic acid.

![Fig. 3. Isoelectric focusing of intact and Tween-ether disrupted AKR virus in a pH gradient of 3 to 10.](a) Purified AKR virus (3.2 ml.) containing 0.1% ampholine was applied to the column at a position corresponding to fraction 30. Electro-focusing was done at a potential of 300 v for 5' for 24 hr. Sixty-nine fractions of 1.7 ml. each were collected. The localization of virus measured is indicated by CF reactions with M-MSV rat serum. The apparent isoelectric point of the virus is 4.0. $E_{280}$ ■ pH ○ ○. CF solid line. (b) Purified virus was disrupted with Tween-ether by Procedure (a) (see Methods). 8.2 ml. clarified sample with 1.0% Ampholine was applied to the column corresponding to fractions 29 and 30. Electrofocusing was done as above. Seventy fractions of 1.7 ml. each were collected. gs antigen is localized with an isoelectric point of 6.7 (reactions with M-MSV rat serum) and envelope antigens are found in the acidic region with the main component at pH 4.5 (reactions with AKR rat antisemum and M-MSV rat serum). Symbols as in (a). (c) The same isoelectric focusing run as depicted in (b). Fractions were tested with guinea-pig antiserum to purified Rauscher virus gs antigen. The peak of CF activity was obtained at pH 6.7. Symbols as in (a). (d) Purified [H]leucine-labelled virus was disrupted and electrofocused as above for 36 hr. Sixty fractions of 2 ml. each were collected. A sample (0.5 ml.) of each fraction was precipitated by adding 1 ml. 10% TCA. Precipitates were collected on Millipore filters (0.45 μm.) and counted in Bray's solution. Peaks at 4.3 and 6.7 correspond to envelope and gs antigens respectively. Radioactivity, solid line, pH ○ ○.}

Three major extinction peaks appeared at lower pH values (Fig. 3b, c) with apparent isoelectric points of 3.6 (fraction 15), 4.1 (fraction 19) and 4.5 (fraction 22) respectively. The third peak (isoelectric point pH 4.5) corresponded to a CF antigen peak as detected by m-MSV rat and AKR rat serum. This blocked virus specific neutralizing antibody and antibody produced against it neutralized the focus forming activity of the Gross pseudotype of murine sarcoma virus. The sum of this data suggests that this probably represents an ether resistant envelope antigen.
Fig. 4. Immunoelectrophoresis of the gs antigen of AKR leukemia virus. The cathode is to the right of this figure. Precipitin patterns were developed with m-MSV rat serum. Well 1: Virus partially disrupted by 10 min. of Tween-ether extraction; at least two immunologically identical components are present. Well 2: The same preparation as no. 1 but exhaustively extracted with Tween-ether; only the cathodal migrating component remains. Well 3: Disrupted virus preparation used for electrofocusing. Well 4: gs antigen after isoelectric focusing (isoelectric point 6·7 component).

Table 2. Purification of murine C-type virus group-specific antigen

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Volume (ml.)</th>
<th>Total (titre x ml.) CF units*</th>
<th>Specific activity CF units/mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified virus</td>
<td>17</td>
<td>272†</td>
<td>34</td>
</tr>
<tr>
<td>Tween-ether—disrupted virus</td>
<td>35</td>
<td>1120</td>
<td>168</td>
</tr>
<tr>
<td>Waterphase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waterphase supernatant—after 100,000g, 1 hr centrifugation</td>
<td>35</td>
<td>1120</td>
<td>235</td>
</tr>
<tr>
<td>Isoelectric focused gs antigen</td>
<td>7·5</td>
<td>1344§</td>
<td>3072</td>
</tr>
</tbody>
</table>

* Using gs specific guinea-pig antiserum as 4 units.
† Tested at the same time as the purified antigen, after standing at 5° for several days. Virus tested immediately after purification regularly gave titres 4 to 8 fold lower than these.
§ Summation of CF units recovered in 5 individual fractions in the gs peak.

DISCUSSION

The isoelectric focusing procedure has proved extremely useful for purification of the major group-specific antigen of murine C-type viruses (Gregoriades & Old, 1969). The antigen was obtained as a homogeneous protein, free of nucleic acid and in immunogenic form. The difference in isoelectric point value obtained by us (6·7), and Gregoriades & Old (1969) (5·6) is readily attributable to the relative lack of RNA in our preparations, at least tenfold less than these authors reported. The reason for this difference in RNA content is
Fig. 5. Electrofocusing of [3H]uridine labelled AKR virus on a pH gradient of 5 to 8 after disruption with Tween-ether. 4.7 ml. sample was applied to the column at a position corresponding to fraction no. 30. Electrofocusing was at 700 V, 5° for 64 hr. Sixty-one fractions, 2 ml. each, were collected; 0.2 of each fraction was counted in 10 ml. Bray's solution. CF titre reciprocal with M-MSV rat serum (■—■), [3H]uridine activity (○—○), pH (▲—▲).

not immediately obvious; however, it is clear that the same antigenic component was obtained in each laboratory (based on exchange of reagents). Recovery of antigen by the electrofocus procedure was virtually 100% of the input, in contrast to the low yields obtained by gel filtration (Schäfer et al. 1969). Thus this seems to be the method of choice for purification of the gs antigen from other C-type viruses. While our results tend toward homogeneity, there are now several reports of multiple gs components in both avian and murine C-type viruses (Geering et al. 1966; Gregoriades & Old, 1969; Armstrong, 1969; Roth & Dougherty, 1969). In most cases it is not possible to say whether or not these are truly immunologically distinct entities or are artifacts resulting from incomplete extraction, reaggregation, or association with lipid or other components. The existence of several physically distinct, immunologically identical species of the murine gs-1 antigen has been noted (Gregoriades & Old, 1969) and also observed by us under certain extraction conditions. Until monospecific sera are available for each of the ‘immunologically distinct’ components, some reservation as to the true multiplicity of antigens should be maintained. In at least one case, however, the finding of a shared antigen between murine and feline C-type viruses (not gs-1) is potent evidence in favour of more than one internal antigen in these viruses (Geering et al. 1968). The results obtained with isoelectric focusing of amino acid-labelled-disrupted virus indicate that the gs-1 component is the major virus component with an isoelectric point near neutrality, thus implying that the other internal components are structurally quite different and perhaps occupy a different physical location in the intact
virion. Preliminary studies with other species C-type viruses have also shown 1 to 2 major radioactive peaks in the neutral to basic range. This allows a presumptive gs antigen to be identified in the absence of serological reagents. We expect this to be extremely useful for preparations of gs antibody for newly isolated C-type viruses.

Although C-type viruses are ether-sensitive, certain envelope antigens appear to be ether-resistant and thus subject to purification procedures. At least one fraction active in inducing neutralizing antibody has been isolated by isoelectric focusing. Characterization of this component is currently under study.

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REFERENCES


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