Interaction of Non-Specific Inhibitor and Rabies Virus Haemagglutinin

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SUMMARY

In order to elucidate the properties of an inhibitor of rabies virus haemagglutinin in normal animal sera, experiments were made with the HEP Flury strain and calf serum which contains the inhibitor. The results of physico-chemical treatment, gel-filtration and density analysis suggested lipoprotein involvement. When inhibitor and haemagglutinin were mixed, the separate activities could be recovered from the mixture by centrifuging on a sucrose density gradient. By contrast, neither haemagglutinin nor inhibitor could be recovered by this treatment when the inhibitor was added at the start of virus growth. The binding of inhibitor with rabies virus during virus growth seems irreversible and different from the binding of inhibitor with pre-formed rabies haemagglutinin.

INTRODUCTION

It is known that normal human and animal sera contain a non-specific inhibitor to rabies virus haemagglutinin and that haemagglutinating activity of virions is not demonstrable when rabies virus is grown in the presence of this inhibitor (Halonen et al. 1968; Kuwert et al. 1968). Recently, Halonen, Toivanen & Nikkari (1974) reported that most serum inhibitor was found in the lipoprotein fraction. However, little is known about the mode of action of the inhibitor. Our present report deals with attempts to understand the interaction between non-specific inhibitor and haemagglutinin during virus replication. Some characteristics of the non-specific inhibitor are also described.

METHODS

Virus. The HEP Flury strain of rabies virus was grown in primary chick embryo fibroblast (CEF) cells.

Preparation of virus. Virus was grown in CEF cells as described by Kondo (1965). Monolayers of CEF cells grown in EYL supplemented with 5% of calf serum (CS) were washed once with phosphate buffered saline (PBS) and incubated with PBS for 60 min at 37 °C. The PBS was then discarded and the cells infected with rabies virus at multiplicities of infection between 0.01 and 0.1. After 60 min at 37 °C, fresh 199 medium was added to the cultures, with or without 1% CS, and incubated at 35 °C. After 6 days the culture fluid was collected and centrifuged at 600 g for 5 min to remove cell debris, and polyethylene glycol
PEG) 6000 was added at a final concentration of 6% in 0.5 M-NaCl. After stirring overnight at 4 °C, the precipitate was collected by centrifugation at 3300 g for 20 min at 4 °C, resuspended in TNE buffer (0.05 M-tris, 0.14 M-NaCl and 5 × 10^-3 M-EDTA at pH 8.0; abbreviated as TN buffer when EDTA was omitted) and used as the virus preparation concentrated to 100 times the original culture fluid.

**Plaque titration.** Infectivity determinations were made by plaque titration as described by Kondo, Takashima & Suzuki (1972).

**Haemagglutination (HA) and haemagglutination inhibition (HI) tests.** HA and HI tests were performed by the procedure of Halonen et al. (1974) except that in the HI test, 4 units of haemagglutinin were used instead of 2 units as they described. The highest dilution of the original, undiluted sample of serum or inhibitor which caused complete inhibition of agglutination was noted and its reciprocal was taken as the haemagglutination inhibition activity (units-H.I.U.).

**Treatment of sera with acetone or kaolin.** Sera were treated with acetone or kaolin following the method described by Clark & Casals (1958) for arboviruses and that described by Halonen, Toivanen & Nikkari (1974) for rabies virus. Kaolin (acid washed, Fisher Scientific Co., Fair Lawn, N.J., U.S.A.) was added to the serum diluted 1 to 10 with borate buffered saline, pH 9.0.

**Sephadex G-200 gel filtration.** A column of Sephadex G-200 was equilibrated with a solution containing phosphate buffered saline pH 7.2, and 0.7 ml of serum sample was applied. Gel filtration was performed using the above buffer in the cold in a 1.0 × 50 cm column and 0.5 ml fractions were collected. After determination of extinction at 280 nm each sample was examined for HI activity.

**Ultracentrifugal fractionation of serum lipoproteins by flotation.** The stock salt solution of sodium chloride and potassium bromide (ρ = 1.346 g/ml) was added to serum to obtain the density as described by Havel, Eder & Bragdon (1955) and Halonen et al. (1974). The mixture of calf serum and the above stock salt solution was centrifuged at 105000 g for 20 h at 15 °C in a Spinco no. 40 rotor. Fractions (1 ml) were collected from the bottom of the centrifuge tube and assayed for HI activity.

**Separation of inhibitor from inhibitor-virus complex.** One ml of virus having a HA titre of 1:64 was mixed with an equal volume of a diluted calf serum and held at 4 °C for 2 h or 19 h or at 37 °C for 2 h. The mixtures were then centrifuged at 65000 g for 60 min at 4 °C in a Spinco no. 40 rotor. The resulting pellets were suspended with 1 ml of TN buffer (pH 7.8). HA and HI activities in the pellet suspension, supernatant fluid and the mixture which had not been centrifuged were assayed. In another experiment, a mixture of virus suspension containing HA and calf serum was held at 4 °C for 16 h in the presence or absence of EDTA (0.03 M). The mixtures were then centrifuged through a 10 to 40% (w/v) linear sucrose density at 65000 g for 2 h in a Spinco SW 50 L rotor at 4 °C and each fraction assayed for HA and HI activity.

**RESULTS**

Some properties of rabies virus haemagglutinin inhibitor obtained from the sera of various species of animal

Normal sera from various animals such as calf, rabbit, mouse, rat and guinea pig were found to have high titres (1:10240) of non-specific haemagglutinin inhibitor. These inhibitory activities were hardly altered by heating at 56 °C for 30 min or acetone treatment but were removed by kaolin treatment. To examine the nature of the non-specific inhibitor,
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Fig. 1. HI activity of normal calf serum in the fractions obtained by Sephadex G-200 gel filtration.

- - Extinction at 280 nm; O -- O, HI activity.

we tested the HI activity of normal calf serum in the fractions obtained by Sephadex G-200 gel filtration. Inhibitory activity was recovered in the areas of the first two protein peaks (Fig. 1). The first was present in the void volume fraction which contained serum lipoprotein and the second was eluted in the fractions containing immunoglobulins, though the main inhibitory activity was found in the first peak.

To confirm the lipoprotein nature of the non-specific inhibitor, we examined HI activity of calf serum after ultracentrifugal fractionation of serum lipoproteins by flotation in a medium of graded density. As shown in Fig. 2, HI activity was recovered in the low density lipoprotein fraction ($\rho = 1.006$ to 1.063).

**Effect of non-specific inhibitor on rabies virus haemagglutinin production**

It is known that rabies virus haemagglutinin is demonstrable only when serum free medium is used for virus growth (Halonen *et al.* 1968; Kuwert *et al.* 1968).

To examine the effect of inhibitor on the production of the haemagglutinin during the growth of rabies virus in CEF cells, serum from which the inhibitor had been removed was
Fig. 2. Ultracentrifugal fractionation of serum lipoproteins by flotation. Fractions (1 ml) were collected from the bottom of the centrifuge tube and assayed for HI activity (○—○). (a) \( \rho = 1.066 \), (b) \( \rho = 1.019 \), (c) \( \rho = 1.063 \) and (d) \( \rho = 1.21 \).

Table 1. Infectivity and HA activity of rabies virus grown in CE cells in the presence or absence of calf serum*

<table>
<thead>
<tr>
<th>Serum†</th>
<th>Activity</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture fluid</td>
<td>Carbowax precipitated</td>
<td>Culture fluid</td>
</tr>
<tr>
<td>−</td>
<td>p.f.u./0.2 ml</td>
<td>2.0 ( \times 10^4 )</td>
<td>2.7 ( \times 10^4 )</td>
</tr>
<tr>
<td>+</td>
<td>H.A.U./0.025 ml</td>
<td>&lt; 1</td>
<td>256</td>
</tr>
<tr>
<td>Untreated</td>
<td>H.A.U.</td>
<td>7.5 ( \times 10^4 )</td>
<td>8.0 ( \times 10^5 )</td>
</tr>
<tr>
<td>+</td>
<td>p.f.u.</td>
<td>&lt; 1</td>
<td>16</td>
</tr>
</tbody>
</table>

* Chicken embryo cells were infected as described in Methods. Infected culture fluid was harvested on 6th day after infection. Samples were then assayed for infectivity (p.f.u.) and haemagglutination (H.A.U.) in chicken embryo cells.
† −, No serum; +, with serum.
‡ Not tested.

used in the medium. After adsorption of the virus to CEF cells, 1% kaolin-treated serum (KCS) was added to the fresh medium and the cultivation was continued as described in Methods. As shown in Table 1, HA activity was present in virus harvest grown in the presence of KCS and both HA activity and infectivity were comparable with those of control, serum free (CS−) cultures. On the other hand, virus grown in the medium supplemented with 1% non-treated calf serum (CS+) showed no HA activity though the infectivity was higher than that found in fluids of the supernatant from KCS+ or CS− cultures. These results suggested that the inhibitor was masking haemagglutinin components of virions or inhibited haemagglutinin production itself.
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Table 2. *Recovery of HA and HI activities by centrifugation after binding*

<table>
<thead>
<tr>
<th>Addition of calf serum</th>
<th>Incubation</th>
<th>HI activity</th>
<th>HA activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before centrifugation</td>
<td>After centrifugation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Supernatant</td>
<td>Precipitate</td>
</tr>
<tr>
<td>+</td>
<td>37 °C 2 h</td>
<td>32*</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>4 °C 2 h</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>4 °C 19 h</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>−</td>
<td>37 °C 2 h</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
</tr>
<tr>
<td></td>
<td>4 °C 2 h</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
</tr>
<tr>
<td></td>
<td>4 °C 19 h</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

* Titre per 0.025 ml.

**Fig. 3. Interaction of normal calf serum and rabies virus in the presence or absence of EDTA.** HA and HI activity after sucrose density gradient centrifugation. HA activity with (-----) or without (-------) EDTA and HI activity with (○—○) or without (●—●) EDTA.

**Separation of inhibitor from inhibitor-virus complex**

It was considered important to determine whether the inhibitor or haemagglutinin could be recovered from a mixture of the two in order to show the mechanism of their interaction. The virus was mixed with the calf serum, incubated and sedimented at a high speed as described in Methods. HA and HI activities in the pellet suspension and supernatant and the mixture before centrifugation were assayed. As shown in Table 2 no HA activity could be recovered in the pellets or the supernatants after treatment with CS. On the other hand, HI activity was detected in the supernatants. In the case of the CS− control, HA activity was recovered in the pellets and in the supernatant but mostly sedimented with the virus. In another experiment, a virus-serum mixture was held in the presence or absence of EDTA and...
Fig. 4. Effect of EDTA on rabies virus grown in the presence of untreated or kaolin-treated calf serum. Determination of HA activity after sucrose density gradient centrifugation. (a) HA activity in the presence of untreated calf serum with 0.003 M (▲—▲), 0.01 M (□—□), 0.03 M (■—■) or without (△—△) EDTA. (b) HA activity in the presence of kaolin-treated calf serum with 0.03 M (○—○) or without EDTA (●—●).
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Fig. 5. Production of rabies virus haemagglutinin in the presence (CS+) or absence (CS−) of calf serum. On the first (△—△), second (○—○) and fourth (●—●) day after infection, infected fluid was removed (f.c.), cells were washed with PBS and mixed with the serum free medium and then incubated at 35°C. The HA yields of CS− (□···□) and CS+ (■—■) cultures were plotted as controls. f.c., Change of medium on days 1, 2 and 4.

fractionated by sucrose density gradient centrifugation. EDTA was used following a report (Furukawa et al. 1967) in which rubella HA was successfully recovered from the HA-inhibitor mixture.

The details of the procedure are described in Methods. As shown in Fig. 3, both HA and HI activities were recovered separately, HI activities being at the top of the tube and HA in fractions 5 to 9 at the same position as the free virion, either in the presence or absence of EDTA. These results indicate that the inhibitor reacts with virions by some weak binding force.

Attempt to recover haemagglutinin in the culture fluid of rabies virus grown in association with the inhibitor.

It was expected that in the case of HA negative virus in which HA activity was repressed by the presence of calf serum during virus reproduction, HA activity might be recovered by treatment with EDTA as described in the preceding paragraph. Virus grown in the presence of inhibitor was mixed with various concentrations of EDTA (0.003 M, 0.01 M and 0.03 M), kept at 4°C for 16 h and then centrifuged in 20 to 50% (w/v) linear sucrose gradients at 65,000 g for 2 h in a Spinco SW 50 L rotor at 4°C. No HA activity was recovered in any of the fractions (Fig. 4a). As a control, virus grown in the absence of inhibitor was fractionated
in the same manner after treatment with 0.03 M-EDTA. The distribution pattern of HA activity in both controls with or without EDTA was found to be identical (Fig. 4b).

**Effect of withdrawal of inhibitor on the production of rabies virus haemagglutinin**

The following experiments were performed to examine the effects of the serum inhibitor on the haemagglutinin during the course of virus growth. After the infection of CEF cells with rabies virus, medium 199 supplemented with 1% of calf serum was added and the cells were incubated at 35 °C. On the first, second and fourth day after incubation, the fluids were removed, the cells washed three times with PBS and serum-free medium was added before continuing incubation at 35 °C. Six days after infection, all culture fluids were harvested, concentrated with PEG as described in Methods and assayed for HA activity. As shown in Fig. 5 haemagglutinin was produced in the supernatant fluid after the inhibitor was removed.

**DISCUSSION**

It has been demonstrated previously that rabies virus haemagglutinin is highly sensitive to non-specific serum inhibitors in normal animal sera which can be removed by kaolin treatment (Halonen et al. 1968). They have also shown that this inhibitor was associated with lipoprotein (Halonen et al. 1974) and our results confirm their findings. Our conclusion is based on removal of the inhibitor from normal sera by kaolin-treatment, determination of macromolecule size by gel filtration and density by flotation experiments. β-Lipoprotein from human sera obtained commercially also showed highly inhibitory activity to rabies virus haemagglutinin (data not shown). These results confirm the work of Halonen et al. (1974).

Further investigation was made on the biological activity of the inhibitor, i.e. the mechanism of inhibition of rabies virus haemagglutinin. When the inhibitor was added to virus containing HA no haemagglutinin could be detected in the virus pellet after centrifugation (Table 2). This finding suggested that the serum-inhibitor did not act by blocking the virus receptor site of the goose cells used in the HA titration but had a direct effect on the virus particles. If the HA site of the rabies virus represents an initial site in infection, it is reasonable to suppose that serum inhibitor might have some neutralizing effect on the rabies virus. However, we were unable to demonstrate any neutralizing activity with serum inhibitor by the plaque-reduction test (data not shown).

Our experiments showed that the infectivity of virus grown in the presence of inhibitor was as high as that of virus grown in serum-free medium. These results suggest that haemagglutination-inhibition by the inhibitor may not be due to its binding of an active site in the haemagglutinin. In other experiments, it was demonstrated that the inhibitor could be separated from virus containing haemagglutinin by rate zonal centrifugation, suggesting a loose interaction between the inhibitor and the virion, but these were artificial mixtures of virus containing haemagglutinin and calf serum.

It is known that when rabies virus is grown in a medium supplemented with normal serum, no HA activity can be detected (Halonen et al. 1968). However, as much HA activity was demonstrated with virus grown in KCS medium as with virus grown in CS—medium (Table 1). Furukawa et al. (1967) showed that the addition of calcium or magnesium chloride to the tissue culture medium prevented the release of haemagglutinin of rubella virus. They could recover HA activity from rubella cultured with serum by adding EDTA which acted to remove cations from the medium. In order to recover HA activity from virus
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grown in a medium containing calf serum, we used a similar treatment. However, we were unable to recover HA activity by EDTA treatment or by rate zonal centrifugation as shown in Fig. 4(a).

These results suggested that the interaction of haemagglutinin and the inhibitor during virus growth within the infected cells is different from that observed in the *in vitro* HI system. It was suggested that inhibitor added during the growth cycle of rabies virus might interact with virus particles more intimately than inhibitors added to pre-formed virus. The results with EDTA suggest that divalent cations do not participate in this interaction. Two possible mechanisms of inhibition may be suggested in the first case. One is that inhibitor added during virus growth may be adsorbed to the cell surface or integrated into cell membrane. Thus, virus may be enveloped in the budding process by this inhibitor-containing cell membrane and therefore from non-HA particles. The second possibility is that the structure of haemagglutinin might be affected by the presence of the inhibitor during virus growth. The first possibility is unlikely because the inhibitory serum added in the medium had little or no effect on the later production of haemagglutinin after washing the inhibitors out of monolayer cells (Fig. 5). We have no clear evidence to support the second possibility. Further experiments will be needed to discover what is occurring within the infected cells.

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REFERENCES


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