Effect of Heparin on Hemagglutination by Equine Arteritis Virus

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ABSTRACT. Heparin inhibited hemagglutination (HA) by equine arteritis virus (EAV) as well as did HA by Aujeszky’s disease virus (ADV), but failed to inhibit HA by parainfluenza virus type 3 (PIV-3). The minimal concentration of heparin required to inhibit 8 HA U of EAV was 0.1 U/ml. In addition, most EAV hemagglutinin was retained by heparin acrylic beads, as was ADV hemagglutinin, but was not PIV-3 hemagglutinin. Mouse erythrocytes failed to combine with the HA inhibitory factor of heparin. However, mouse erythrocytes treated with heparinase had greatly reduced agglutinability by EAV. All these findings suggest that a heparin-like molecule on the surface of EAV was 0.1 U/ml (ADV) but failed to inhibit HA by parainfluenza virus type 3 (PIV-3). The minimal concentration of heparin required to inhibit 8 HA U of PIV-3 was 0.1 U/ml (ADV). Therefore, of interest to test the effect of heparin on EAV hemagglutination.

The objective of the present study was to investigate whether heparin had any effect on EAV hemagglutination as observed for the hemagglutination of herpesviruses, some arboviruses and PRRSV mentioned earlier.

MATERIALS AND METHODS

Viruses: The Bucyrus strain of EAV [2], supplied by Dr. W. H. McCollum, University of Kentucky, Lexington, KY., U.S.A., was used. The Indiana S strain of ADV, supplied by the courtesy of Dr. P. Gustafson, Purdue University, West Lafayette, IN., U.S.A., and the BN1-1 strain of parainfluenza virus type 3 (PIV-3) [3] were used as control viruses.

Cell cultures: The continuous cell lines, RK13 cells (ATCC CCL37) from rabbit kidney, CPK cells from pig kidney and MDBK cells from bovine kidney were used. These cells were grown and maintained by the method described in the previous paper [1].

Erythrocytes: Mouse erythrocytes (ddY strain) obtained in Alsever’s solution were washed three times with phosphate buffered saline (PBS) (0.15 M NaCl, 0.02 M phosphate buffer, pH 7.2), and 0.3% erythrocyte suspension in a diluent consisting of PBS, 0.2% BSA and 0.1% gelatin was used for hemagglutination (HA) with EAV, ADV and PIV-3.

Heparin, heparin acrylic beads and heparinase: Heparin (from porcine intestinal mucosa, Product No. H-5263, Sigma Chemical Co., St. Louis, MO., U.S.A.), heparin acrylic beads (No.5263, Sigma Chemical Co., St. Louis, MO., U.S.A.), and heparinase (from Flavobacterium heparium, Product No. H-2519, Sigma Chemical Co., St. Louis, MO., U.S.A.) were used.

Preparation of hemagglutinin: EAV hemagglutinin was prepared from the RK13 cells infected with the Bucyrus strain of EAV by the method described previously [7] with the slight modification noted below. Briefly, the RK13 cell monolayers in 500 ml bottles were infected with 10^3 TCID_{50} of EAV. After 1 hr adsorption at 37°C, the monolayers were washed once with Eagle’s minimum essential medium supplemented with 0.5% lactalbumin hydrolysate, 0.2% bovine serum, 0.12% NaHCO_{3}, and antibiotics (MM) fed with 30 ml of MM and incubated at 37°C until cytopathic effect (CPE) was present in about 70% of the monolayer(approximately 3 days postinfection). The infected RK13 cells were scraped off from the bottle surface with a rubber policeman. The cell-medium mixture was
centrifuged at 2,000 x g for 20 min. The resultant pellet was resuspended in 1/100 the original volume of PBS containing 0.2% BSA. The suspension was subjected to 3 cycles of freeze-thaw treatment and centrifuged at 2,000 x g for 20 min. The supernatant was pretreated with Tween 80 (polyoxyethylene sorbitan monooleate, Sigma p-8074) at a final concentration of 0.06% (v/v) for 15 min followed by treatment with 50% (v/v) of ether (Diethyl ether, Wako 055–01155; Wako Pure Chemical Co., Osaka, Japan) for 15 min. These treatments were carried out with continuous shaking in ice bath. The treated material was centrifuged at 2,000 x g for 20 min, and the Tween-ether phase was sucked off and discarded. The resultant aqueous phase after elimination of the residual ether by cautiously bubbling nitrogen gas through the sample was used as a HA antigen.

ADV and PIV-3 hemagglutinins were prepared from the infected culture fluids of CPK and MDBK cells infected with ADV and PIV-3, respectively, by the method described in the previous papers [3, 5].

HA and HA-inhibition (HI) tests: HA and HI tests were carried out by a microtiter method using 96-round-well microtiter trays (Sanko Junyaku Co., Tokyo, Japan). For HA tests, serial two-fold dilutions of hemagglutinin in the diluent were prepared in 25 µl of the diluent, and mixed with 50 µl erythrocyte suspension after adding 25 µl of the diluent. The mixtures were incubated at room temperature for 2 hr before the results were read, unless stated otherwise. The HA titer was expressed as the reciprocal of the highest dilution showing complete HA. For HI tests with heparin, serial two-fold dilutions of heparin in 25 µl of PBS were made, then mixed with 25 µl of the diluent containing 8 HA U of hemagglutinin. After incubation at 37°C for 1 hr, 50 µl of mouse erythrocyte suspension was added to the mixtures, which were incubated at room temperature for 2 hr, unless stated otherwise, and the results were read. The well with complete HI was used as the endpoint. Control wells with hemagglutinin and PBS, instead of heparin, were set up, incubated, centrifuged, resuspended, and assayed in a similar manner.

Effect of temperature on HA reaction: Eight HA U of EAV, ADV or PIV-3 hemagglutinin were mixed with various concentrations of heparin, and the heparin-hemagglutinin mixtures were incubated at 4°C, room temperature or 37°C, before adding mouse erythrocyte suspension. These mixtures were used for the HA test.

Effect of pretreatment of mouse erythrocytes with heparinase on HA: One volume of 0.3% mouse erythrocyte suspension was mixed with one volume of 8 HI U of heparin and the mixture was incubated at 37°C for 1 hr. The erythrocytes were washed twice with PBS and used in a 0.3% suspension for the HA test. Controls, comprising erythrocytes and PBS instead of heparin, were set up, incubated, centrifuged, resuspended, and assayed in a similar manner.

Effect of pretreatment of mouse erythrocytes with heparinase on HA: One volume of 0.3% mouse erythrocyte suspension was mixed with one volume of various concentrations of heparinase-0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 4.0 U/ml-and the mixtures were incubated at 37°C for 1 hr. The erythrocytes were in a 0.3% suspension in an HA test with EAV, ADV or PIV-3 hemagglutinin. Control erythrocytes, using PBS instead of heparinase, were set up, incubated, centrifuged, resuspended and assayed in a similar manner.

RESULTS

Effect of heparin on HA by EAV, ADV or PIV-3: The minimal concentrations of heparin required for inhibition of 8 HA U of the Bucyrus strain of EAV and the Indiana S strain of ADV were 0.1 U and 0.1 U, respectively, at all temperatures tested. However, HA by the BNI-1 strain of PIV-3 was not affected at any concentration of heparin tested (Table 1).

Effect of heparin treatment of erythrocytes on HA reaction and attempts to bind heparin to mouse erythrocytes: Mouse erythrocytes treated with heparin were agglutinated by the hemagglutinin of EAV to the same extent as were the

Table 1. Effect of heparin on hemagglutination (HA) by EAV, ADV and PIV-3

<table>
<thead>
<tr>
<th>Final concentration of heparin (U ml⁻¹)</th>
<th>EAV</th>
<th>ADV</th>
<th>PIV-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>0.1</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>0.01</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>0.001</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>0.0001</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Control

a) Eight HA U of hemagglutinin were mixed with heparin, and the mixtures were incubated at 37°C for 1 hr, then were used for the HA test.
b) PBS instead of heparin was mixed with hemagglutinin.

+ = inhibition; = no inhibition.
untreated control cells. No evidence of significant binding of heparin to mouse erythrocytes was observed (data not shown).

**Binding of EAV hemagglutinin to heparin acrylic beads:** The hemagglutinins of EAV and ADV bound to heparin acrylic beads, but the hemagglutinin of PIV-3 did not bind (Table 2).

**Effect of HA of pretreatment of mouse erythrocytes with heparinase:** Erythrocytes pretreated with heparinase had greatly reduced HA titers with EAV and ADV hemagglutinins. In contrast, heparinase pretreatment of mouse erythrocytes had no effect on the titer of the PIV-3 hemagglutinin (Table 3).

**DISCUSSION**

Our results from the present paper indicate that heparin inhibited HA by EAV as well as by ADV [13] and PRRSV [6], but failed to inhibit HA by PIV-3. This finding indicates that heparin acts selectively, inhibiting HA by EAV.

No evidence for interaction of heparin with mouse erythrocytes was obtained, indicating that heparin inhibited the hemagglutinating activity of EAV by direct interaction with its hemagglutinin, rather than by interaction with the erythrocytes. These results do appear to corroborate our findings that growth of EAV is inhibited by heparin [1], therefore, it is possible that the cellular receptor for EAV hemagglutinin is a heparin-like molecule.

In addition, heparinase treatment of mouse erythrocytes reduced the binding of EAV hemagglutinin to the cell, and rendered the cell resistant to agglutination. These findings indicate that a heparin-like molecule on the surface of mouse erythrocytes serves as the HA receptor.

All our findings indicate that the effect of heparin on EAV hemagglutinin with mouse erythrocytes is attributable to an effect on the hemagglutinating activity, not on the erythrocytes. Our findings indicate that heparin may bind to EAV hemagglutinin, presumably occupying the sites necessary for attachment of the hemagglutinin to erythrocytes as well as those of PRRSV [5], herpesviruses [13], and bunyaviruses [6].

The inhibitory action of heparin has practical importance in several procedures commonly used by virologists. Because all our findings, together with our previous results [1], demonstrated that EAV infection was inhibited by heparin, heparinized blood must be regarded as not an ideal specimen for the virus isolation attempts.

Further detailed studies are necessary to compare heparin activities against the EAV hemagglutinin with those of PRRSV, herpesvirus and bunyavirus hemagglutinins and to elucidate the mechanism of the inhibitory activity of heparin on EAV hemagglutinin.

**REFERENCES**