Synergistic Neutralization of Rubella Virus by Monoclonal Antibodies to Viral Haemagglutinin

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SUMMARY

Using murine monoclonal antibodies (MAbs) to rubella virus haemagglutinin, five epitopes were identified in competitive ELISA binding assays: A, B, D and E by haemagglutination-inhibiting (HI) MAbs with no neutralizing (Nt) activity, and C by a MAb with neither activity. However, when HI and Nt activities were determined in the presence of anti-mouse immunoglobulins, epitopes A, B and D were defined by both HI and Nt MAbs, whereas epitopes C and E were identified by HI MAbs without Nt activity. A synergistic Nt activity, in the absence of anti-mouse immunoglobulins, was displayed by mixtures of antibodies of different epitope groups. Analysis of mixtures of MAb pairs each belonging to a different epitope class, showed that synergistic Nt activity was elicited primarily by the group A epitope, secondarily by groups B and D and only minimally by groups C and E.

Rubella virus is a non-arthropod-borne member of the family Togaviridae (Porterfield et al., 1978), the only known member of the genus Rubivirus (Horzinek, 1981). It has recently been shown to contain three major structural proteins: E1, E2 and C (Oker-Blom et al., 1983; Waxham & Wolinsky, 1983, 1985). Monoclonal antibodies (MAbs) reactive to the E1 glycoprotein of rubella virus inhibit haemagglutination (HA) and/or block virus infectivity (Ho-Terry et al., 1984; Waxham & Wolinsky, 1983). Recently, using a panel of MAbs and competitive binding assays, six distinct epitopes have been identified within the E1 glycoprotein of rubella virus (Waxham & Wolinsky, 1985).

In the present report, using a panel of 10 murine MAbs all reactive to the E1 glycoprotein by immunoblotting, five distinct epitopes were similarly identified by enzyme-linked immunosorbent assay (ELISA) competitive binding assays (CBAs). However, when MAbs were tested by haemagglutination-inhibition (HI) and by neutralization (Ni) in the presence of anti-mouse immunoglobulins (anti-Ig-assisted HI and Ni), the five epitope groups were clustered in two functional groups, one reactive with MAbs showing both HI and Ni activities, and the other reactive only with HI, but not with Ni MAbs. In the absence of anti-mouse immunoglobulins (i.e. unassisted) Ni activity was detected only when two MAbs reactive to different epitope groups were pooled, and it correlated with the epitope group of the MAb pair tested.

Vero cells, obtained from the American Type Culture Collection (ATCC) (Rockville, Md., U.S.A.), were used for propagation of the Gilchrist and Putnam strains of rubella virus. The cells were grown in Eagle's minimum essential medium (EMEM) with 10% foetal calf serum (FCS). Virus from infected culture medium was concentrated approximately 100-fold using a hollow fibre apparatus (Amicon) and purified first by centrifugation through one discontinuous and one linear sucrose gradient, and then through a linear gradient of 10 to 30% (w/w) potassium...
tartrate containing 10% (v/v) glycerol (Gravell et al., 1977). Banded virus was collected, stored at 
-80 °C, and used for mouse immunization (Putnam strain), ELISA (both strains), SDS–PAGE
and immunoblotting (Gilchrist strain).

Four-week-old BALB/c mice were immunized by intraperitoneal injection of 100 HA units
per mouse of purified rubella virus in Freund's incomplete adjuvant. Four identical doses were
administered after 15, 30, 60 and 120 days. Finally, 400 HA units per mouse in saline were
administered intravenously 4 days before fusion. Immune spleen cells were fused with NS-1
myeloma cells (ATCC) using 50% polyethylene glycol 1500 (ATCC) in RPMI 1640. Rubella
antibody-secreting hybrids were detected by ELISA and, when of interest, cloned twice by
limiting dilution and expanded. Ascites were obtained according to a standard procedure. The
isotype of MAbs was determined by double immunodiffusion against a set of goat antisera
directed to mouse immunoglobulin subclasses (Meloy, Springfield, Va., U.S.A.). Monoclonal
antibodies were purified from ascitic fluids by the caprylic acid (octanoic acid) method
(Steinbuch & Audran, 1969). The protein concentration was determined by measuring the
absorbance of each sample at 280 nm. Immunoglobulins were then coupled to horseradish
peroxidase (Sigma) by the periodate method (Wilson & Nakane, 1978).

The ELISA was a modification of the assay described by Wolinsky et al. (1982). The CBAs
were performed using serial 10-fold dilutions of each unlabelled antibody to which the optimal
dilution of each peroxidase-labelled antibody was added. A 100 µl aliquot of each mixture was
then added to purified virus-coated wells of an ELISA microtitre plate and the test performed as
above.

The HI test was performed according to a standardized procedure (Palmer et al., 1970). In
anti-Ig-assisted HI tests (Umino et al., 1985) the IgG fraction of rabbit anti-mouse
immunoglobulins was added to antigen–antibody mixtures (previously incubated for 30 min at
4 °C) to a final concentration of 1:40 and incubation was continued for 30 min at 4 °C before
addition of erythrocyte suspension.

For the Nt assay, 10-fold dilutions of ascitic fluids in EMEM with 2% FCS were made in
duplicate in sterile 96-well microtitre plates (50 µl/well) and mixed with equal volumes of a
rubella virus dilution containing 50 to 100 p.f.u. After incubation for 1 h at 37 °C, 50 µl of each
mixture were inoculated onto each of two monolayers of Vero cells in microtitre plates. After a
72 h incubation at 37 °C, the cultures were fixed with ethanol and the number of plaques was
determined by the immunoperoxidase staining of the monolayers with MAbs (Gerna, 1975). A
linear relationship between the virus inoculum and the number of plaques was found. In anti-Ig-
assisted Nt tests, 5 µl of the IgG fraction of rabbit anti-mouse immunoglobulins (Dakopatts,
Copenhagen, Denmark) was added to virus–antibody mixtures previously incubated at 37 °C
for 30 min and incubation continued for an additional 30 min before inoculation (Ashe &
Notkins, 1966; Sato et al., 1979).

SDS–PAGE under reducing conditions was performed as reported by Laemmli (1970).
Proteins were then transferred onto nitrocellulose paper according to Burnette (1981).
Immunological detection of transferred proteins was performed by indirect immunoperoxidase.
The immunoblotting technique using a pool of convalescent-phase sera from patients with acute
rubella, detected three major structural polypeptides of purified rubella virus (Fig. 1). These
have relative mol. wt. of 60K (E1, range 58K to 62K), 46K (E2, range 42K to 50K) and 38K (C,
range 37K to 39K). In addition, a minor component was also consistently detected with
approximate mol. wt. 120K, probably a dimer of E1. All the MAbs tested showed a degree of
reactivity with E1, while a weak reactivity with the E1 dimer and also with a minor band
corresponding to about 37K (probably a breakdown product of E 1) was consistently observed.

Results of ELISA CBAs are summarized in Table 1, where five different epitopes appear to be
detected on E1 by the MAbs tested. Epitope A was defined by two antibodies, 7A6 and 6D2.
Epitope B was identified by several antibodies (2C3, 3A4, 3C3 and 8C1). Epitope C was
detected only by antibody 4A6, epitope D by two antibodies, 3A2 and 6D6, and epitope E only
by antibody 4C5. Interestingly, MAbs of groups B and D enhanced binding of MAbs of the
other epitope groups. This further supports the conclusion that the MAbs involved reacted with
distinct epitopes (Lubeck & Gerhard, 1981). Each MAb of the panel was tested for its ability to
Competitive binding assays of peroxidase-labelled MAbs to E1 glycoprotein of rubella virus with ascitic fluids

<table>
<thead>
<tr>
<th>Labelled MAb</th>
<th>7A6</th>
<th>6D2</th>
<th>2C3</th>
<th>3A4</th>
<th>3C3</th>
<th>8C1</th>
<th>4A6</th>
<th>3A2</th>
<th>6D6</th>
<th>4C5</th>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6D2</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2C3</td>
<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>3A4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>3C3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
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<td>±</td>
</tr>
<tr>
<td>8C1</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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</tr>
<tr>
<td>4A6</td>
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<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>3A2</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>6D6</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>±</td>
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</tr>
</tbody>
</table>

* +, >80% competition; ±, 20 to 80% competition; -, <20% competition; 0, >30% enhanced binding of labelled antibody.

Properties of MAbs to E1 glycoprotein of rubella virus

<table>
<thead>
<tr>
<th>MAb*</th>
<th>Ig class</th>
<th>Epitope group</th>
<th>Antibody titre†</th>
<th>HI</th>
<th>A-HI</th>
<th>Nt</th>
<th>A-Nt</th>
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<tbody>
<tr>
<td>7A6 (21-0)</td>
<td>G1</td>
<td>A</td>
<td>20480</td>
<td>160000</td>
<td>&lt;10</td>
<td>10000</td>
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</tr>
<tr>
<td>6D2 (36-0)</td>
<td>G1</td>
<td>A</td>
<td>40960</td>
<td>160000</td>
<td>&lt;10</td>
<td>10000</td>
<td></td>
</tr>
<tr>
<td>2C3 (17-5)</td>
<td>G2a</td>
<td>B</td>
<td>40960</td>
<td>80000</td>
<td>&lt;10</td>
<td>10000</td>
<td></td>
</tr>
<tr>
<td>3A4 (30-0)</td>
<td>G1</td>
<td>B</td>
<td>2560</td>
<td>5120</td>
<td>&lt;10</td>
<td>10000</td>
<td></td>
</tr>
<tr>
<td>3C3 (16-0)</td>
<td>G1</td>
<td>B</td>
<td>640</td>
<td>2560</td>
<td>&lt;10</td>
<td>10000</td>
<td></td>
</tr>
<tr>
<td>8C1 (15-0)</td>
<td>G1</td>
<td>B</td>
<td>640</td>
<td>5120</td>
<td>&lt;10</td>
<td>10000</td>
<td></td>
</tr>
<tr>
<td>4A6 (5-0)</td>
<td>G2b</td>
<td>C</td>
<td>&lt;80</td>
<td>80000</td>
<td>&lt;10</td>
<td>&lt;100</td>
<td></td>
</tr>
<tr>
<td>3A2 (13-0)</td>
<td>G1</td>
<td>D</td>
<td>40960</td>
<td>160000</td>
<td>10</td>
<td>3000</td>
<td></td>
</tr>
<tr>
<td>6D6 (7-5)</td>
<td>G1</td>
<td>D</td>
<td>20480</td>
<td>40960</td>
<td>&lt;10</td>
<td>10000</td>
<td></td>
</tr>
<tr>
<td>4C5 (4-0)</td>
<td>G1</td>
<td>E</td>
<td>5120</td>
<td>10240</td>
<td>&lt;10</td>
<td>&lt;100</td>
<td></td>
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</tbody>
</table>

* Ig concentrations in mg/ml are shown in parentheses.
† HI, haemagglutination-inhibition; A-HI, anti-Ig-assisted HI; Nt, virus neutralization; A-Nt, anti-Ig-assisted Nt.

inhibit the HA activity and neutralize the infectivity of rubella virus (Table 2). An unassisted HI activity was shown by MAbs belonging to all epitope classes, except for class C. The anti-Ig-assisted HI test showed a two- to eight fold increase in HI antibody titres. The only MAb belonging to class C (4A6), which was HI-negative in unassisted assay, became positive at a very high titre when tested for anti-Ig-assisted HI activity. This antibody was the most highly reactive with E1 in the immunoblotting assay. On the other hand, unassisted Nt activity was not shown by any of the MAbs tested. In anti-Ig-assisted Nt assays high antibody titres were shown by MAbs of epitope classes A, B and D, whereas the two antibodies of classes C (4A6) and E (4C5) remained negative.

Mixture of MAbs representative of each epitope group were examined for synergistic activity in an Nt assay where chequerboard combinations of 10-fold dilutions of the two MAbs were tested in the absence of anti-mouse Ig (Fig. 2). Results showed that MAbs belonging to epitope group A displayed Nt activity when associated with antibodies of each of all the other four groups. On the other hand, MAbs of epitope groups B and D showed some Nt activity when associated with each other, or to antibodies of groups C and E. Monoclonal antibodies of epitope groups C and E displayed no Nt activity when combined. This was the only combination of two MAbs from different epitope groups which was completely unable to block virus infectivity.

By CBAs, all our MAbs could be classified into five epitope groups which included two groups of functionally distinct antibodies, differentiated in unassisted HI and Nt assays by the presence
or absence of HI activity together with the lack of any Nt reactivity. However, when we performed HI and Nt assays assisted by anti-mouse immunoglobulins, MAbs of epitope groups A, B and D, possessing unassisted HI activity, acquired Nt activity. Similarly, the MAb of class C, which was HI-negative, acquired high titre HI activity in the anti-Ig-assisted test, although remaining Nt-negative. Only the MAb of the E class did not modify its functional activities in the anti-Ig-assisted assays, remaining HI-positive and Nt-negative. We suggest that all the MAbs directed to E1 glycoprotein of rubella virus might be able to prevent the attachment of virions to erythrocyte receptors with the aid of antiglobulin.

Green & Dorsett (1986) identified at least three epitopes on the E1 glycoprotein of rubella virus by using MAbs with different HI and Nt activities. Waxham & Wolinsky (1985) produced 14 MAbs to E1 which identified six distinct epitopes in CBAs. These epitopes were defined by MAbs which exhibited (i) HI and Nt activities, (ii) HI activity only (three epitopes), (iii) Nt activity only or (iv) neither HI nor Nt activity. In this report, while we did not obtain any MAbs showing Nt activity in the absence of anti-mouse Ig, we could define as many as four distinct epitopes (A, B, D and E) by non-neutralizing MAbs with HI activity. Thus, at least seven different epitopes, four of which were defined by MAbs with HI activity only, could be distinguished on the E1 glycoprotein of rubella virus.

In the present report, we demonstrated that all the MAbs tested, even though lacking an unassisted Nt activity, possessed a latent Nt capacity, which was displayed when two MAbs from different epitope classes were tested in association. However, the epitopes did not appear to be all involved to the same extent in this synergistic Nt activity. The major epitope involved in this phenomenon was epitope A, since MAbs of this epitope group acquired the highest Nt
rubella virus infectivity. Thus, results of tests for synergistic Nt activity appeared to resemble activity; these MAbs had to be mixed with one of the other three groups to display a minimal Nt capacity, which was absent when MAbs of these two epitope groups were associated with one another. Monoclonal antibodies of groups C and E were the only ones which did not neutralize rubella virus infectivity. Both mechanisms have been emphasized in interpreting synergistic Nt of pairs of MAbs to different epitopes of the same viral protein (Clegg et al., 1983; Kingsford, 1984; McCullough, 1986). However, although the synergistic effect of some combinations of MAbs on virus neutralization has already been reported, the extent to which this seems to occur in our rubella virus system appears to be rather unique and warrants further studies.

On the basis of our findings, the antigenic structure of rubella virus E1 might be more complex than previously shown. The unidirectional enhanced antibody binding in CBAs, the synergistic Nt activity of our MAbs and the involvement of multiple epitopes in HI and Nt activities, suggest that the antigenic structure of E1 glycoprotein of rubella virus might be as complex as that of flavivirus E protein (Heinz, 1986).

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Short communication


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