Relationships between Monoclonal Antibody-binding Sites on the Measles Virus Haemagglutinin

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SUMMARY

Twenty-one monoclonal antibodies directed against the measles virus haemagglutinin have recently been obtained. These were known to fall into five groups, each defined by its effects on the biological functions of the H protein. A representative of each group was selected and examined by competitive radioimmunoassay in an attempt to deduce the relationships between antibody-binding sites on the antigen. It was found that these five antibodies formed three binding groups which recognized different but overlapping areas of the molecule. These three areas formed a series of sites which traversed the active region of the H polypeptide. A haemagglutinin-directed monoclonal antibody which displayed a haemolysin-inhibiting activity was also examined. This antibody fitted into the binding group scheme determined here and there was no additional binding site for this molecule on the F protein itself.

INTRODUCTION

The measles virus haemagglutinin is a major structural protein which elicits a strong immune response in any infection associated with this agent. Anti-haemagglutinin antibodies neutralize measles virus, bind to the membranes of lytically and persistently infected cells and inhibit measles virus-mediated haemagglutination. The immune response against this virus protein plays a major role both in overcoming virus infection and preventing reinfection. In contrast to other viruses, such as the orthomyxoviruses (Laver & Webster, 1968), measles virus haemagglutinin (H) has been found to be a relatively stable antigen, and no major differences between the H polypeptides of different measles virus isolates have been observed (Majer, 1972). However, with the availability of hybridoma antibodies, antigenic differences have now been detected (Trudgett et al., 1981; ter Meulen et al., 1981; Birrer et al., 1981a, b) and it could be shown that different strains of measles virus share common antigenic determinants, but also possess distinct antigenic differences in the H polypeptide. Moreover, antigenic change was noted in this protein during the course of a non-productive, subacute sclerosing panencephalitis virus persistent infection (ter Meulen et al., 1981). In a previous report (ter Meulen et al., 1981) we have described twenty-one hybridoma antibodies which were tested and arranged into five biological groups on the basis of their abilities to inhibit haemagglutination and haemolysis or to neutralize virus. In the present study we have further elucidated the physical relationships of binding sites recognized by representatives of this panel of hybridoma antibodies by using a competition radioimmunoassay (Stone & Nowinski, 1980). In this way information about the topographical arrangement of epitopes on the haemagglutinin molecule was obtained. Our results show that these sites comprise a series of three overlapping areas which may traverse the active region of the protein. The relationships between these binding sites should provide a firm basis for further analysis of different measles virus strains and for antigenic change within one strain.

METHODS

Growth of cells, measles virus Edmonston and the production of ascites fluids containing hybridoma antibodies have been described elsewhere (ter Meulen et al., 1981). The assay systems used to determine haemagglutination
inhibition (HI), neutralization (NT) and haemolysin inhibition (HLI) activities were performed as described by Norrby & Gollmar (1975).

**Relative concentrations of hybridoma antibodies.** The relative concentrations of H protein-specific antibody contained in the ascites fluids were determined by the technique of single radial immunodiffusion. Circular wells were cut in 0.5% Litex agarose/phosphate-buffered saline (PBS) gels containing 10 μg/ml of enriched measles virus H protein. Ascites fluids were serially diluted into the wells and allowed to diffuse into the agarose for 24 h at 37 °C. Gels were then washed in PBS for 24 h and stained with Coomassie Brilliant Blue. Precipitin circles whose diameters were linearly related to the ascites fluid dilution were visible. Sizes of these circles were carefully measured and normalized to that obtained for clonal antibody 173.

**Radioimmunoassay (RIA).** The RIA was based on the method of Dörries & ter Meulen (1980). Monoclonal antibodies binding to virus antigens were detected using 121I-labelled anti-mouse F(ab')2 antibodies (ter Meulen et al., 1981). Relative avidities of the hybridoma antibodies used were estimated from the RIA and normalized with respect to antibody 173. Specific counts bound were assumed to be a direct reflection of relative avidity when a constant amount of antigen was saturated with antibody. This method assumes that each antibody binds to only one site per H molecule. RIA curves should level off at the same value for antigen saturation. In practice, differences, assumed to indicate the relative avidity of different monoclonal antibodies, are observed (Stone & Nowinski, 1980; Massey & Schochetman, 1981).

**Labelling of monoclonal antibodies.** To facilitate the investigation of large numbers of hybridoma antibodies, it was decided to use enriched proteins. Accordingly, the mouse ascites fluids were fractionated by three successive ammonium sulphate precipitations using 70%, 80% and 80% (v/v) saturated ammonium sulphate solutions respectively. This procedure was very satisfactory for batch screening and provided a rapid enrichment step. A 200 μl amount of ascites fluid provided sufficient protein for several assays. Enriched antibody preparations were assayed for protein by the method of Lowry et al. (1951) and iodinated using the chloramine T technique (Greenwood et al., 1963). Unincorporated iodine was removed by using pre-formed Sephadex G25M columns PD-10 (Pharmacia).

**Competitive binding assays.** Competition between antibodies was investigated using a procedure modified slightly from Stone & Nowinski (1980). Firstly, that combination of antigen and antibody concentrations which produced sufficient binding for accurate work and which lay in the linear region of binding response was determined. Preformed RIA wells (Dynatech) were coated with measles virus-infected or uninfected Vero cell antigens. Antigen was diluted in PBS to appropriate concentrations and 50 μl of each concentration added per well. Antigens were allowed to bind to the plastic at 37 °C overnight and then non-specific adsorption was blocked with bovine serum albumin [BSA; 5% (w/v) in PBS]. After the coated wells had been washed, iodinated monoclonal antibody was titrated into the wells and the binding of the radiolabel was measured. The competition assay proper was performed by addition of 50 μl of ascites fluid containing the non-labelled (competing) monoclonal antibodies serially diluted in PBS containing 1% BSA. Binding was allowed to proceed for 30 min at 37 °C before addition of the labelled antibody, contained in 20 μl of PBS containing 1% BSA. After a further 60 min at 37 °C, the wells were washed three times with PBS containing 1% BSA and bound radioactivity was measured in a Berthold gamma counter. The reaction was performed in quadruplicate. Maximum possible binding was determined by omitting competitor antibody.

In each competition assay, one reaction using the homologous antibody as a competitor was included to provide a positive control, and two negative controls were also incorporated. These comprised an ascites fluid containing a monoclonal antibody raised against an unrelated (coronavirus) antigen (kindly provided by Dr H. Wege, Würzburg, F.R.G.) and another fluid containing a monoclonal antibody specific for measles virus membrane protein.

Specific binding was measured as the difference between counts bound to wells coated with infected cell antigen and those bound to the same amount of uninfected cell antigens. This value was expressed as the percentage of specific binding in the absence of competitor. Specific counts bound varied with the preparation and ranged between 3000 and 30000 ct/min.

**RESULTS**

One member of each previously defined antibody group was selected for these studies. The antibodies used were all of the IgG class, and the biological properties assigned to them are summarized in Table 1. Another clone from the original hybridoma 26 which displayed similar HI and NT characteristics to group III antibodies was included. This clone also exhibited HLI activity but immunoprecipitated only measles virus H protein. The HLI activity was retained with the HI activity during successive cycles of cloning in soft agar. Togashi et al. (1981) have previously described hybridoma antibodies with similar properties. It was therefore of interest to examine how this antibody might behave in competitive binding experiments. Concentrations of specific antibody in the whole ascites fluid were very similar for each hybridoma preparation examined (Table 1).
Measles haemagglutinin

Fig. 1. Whole ascites fluid 26 (a) and the ammonium sulphate precipitate (b) were analysed by immunoelectrophoresis. Samples were applied to wells (at the positions indicated) cut in 1% agarose-Tris/barbituric acid gels. Electrophoresis was performed for 1 h at 300 V, with the anode to the right. Separated proteins were precipitated with goat anti-mouse whole serum (Dako) and stained with Coomassie Brilliant Blue. The positions of albumin (A) and immunoglobulin (Ig) are indicated.

Table 1. Summary of biological properties of monoclonal antibodies

<table>
<thead>
<tr>
<th>Biological group*</th>
<th>Antibody clone</th>
<th>HI</th>
<th>NT</th>
<th>HLI</th>
<th>Relative avidity†</th>
<th>Relative concn.‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>173</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>&lt;16</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>II</td>
<td>298</td>
<td>128</td>
<td>&lt;8</td>
<td>&lt;16</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>III</td>
<td>585</td>
<td>128</td>
<td>128</td>
<td>&lt;16</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>IV</td>
<td>512</td>
<td>512</td>
<td>&gt;160</td>
<td>&gt;16</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>V</td>
<td>322</td>
<td>16384</td>
<td>1024</td>
<td>&lt;16</td>
<td>2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* Biological group classification and serological titres of hybridoma antibodies used in this study are from ter Meulen et al. (1981).
† Relative avidities were estimated from RIA titration by the method of Massey & Schochetman (1981).
‡ Relative concentrations were estimated by the technique of single radial immunodiffusion and expressed relative to antibody 173.

Properties of antibody preparations

Purity of the ammonium sulphate-precipitated antibodies was assessed by immunoelectrophoresis (Fig. 1). Albumin was largely eliminated and immunoglobulin constituted the major protein present. Following ammonium sulphate enrichment, antibody specificity was preserved since these preparations could still immunoprecipitate the H polypeptide from infected cell lysates (data not shown) and bind in conventional RIA.

Competitive antibody binding

Binding group classification

The results of the competitive binding experiments are given in Fig. 2 and summarized in Table 2. In each case the curves generated fell into two groups, and the presence or absence of competition could be readily determined by reference to the controls.
Fig. 2. The competition radioimmunoassay. Assays were performed as described in the text, using iodinated antibodies 585 (a), 322 (b), 155 (c), 173 (d) and 298 (e). Specific counts bound in the presence of competitor were expressed as a percentage of specific counts bound in its absence, and plotted against the logarithm of competing ascites protein concentration present in each dilution. Individual curves are marked. Competition curves, derived using blank hybridoma (C) and anti-matrix protein (M) hybridoma antibodies (negative controls) are also given. In all cases except antibody 173, triplicate determinations of each point did not differ by more than 10%. In the case of antibody 173 (d), such determinations did not differ by more than 20%.
Fig. 3. Competition radioimmunoassay of antibody 26. Experiments were performed as described in the text, and plotted in the same manner as Fig. 2.

Fig. 4. Binding group analysis. Binding groups 1, 2 and 3 are represented as octagons to illustrate the relationships between the groups. Overlap of octagons indicates positive competition. Size and shape, or extent of overlap of the individual epitopes is not represented.

Table 2. Summary of competition radioimmunoassay results

<table>
<thead>
<tr>
<th>Biological group</th>
<th>Labelled antibody</th>
<th>Competing ascites fluid</th>
<th>Shown in Fig. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>173</td>
<td>+</td>
<td>(d)</td>
</tr>
<tr>
<td>II</td>
<td>298</td>
<td>−* ND†</td>
<td>ND ND</td>
</tr>
<tr>
<td>III</td>
<td>585</td>
<td>− + + + +</td>
<td>(a)</td>
</tr>
<tr>
<td>IV</td>
<td>322</td>
<td>− + + + + +</td>
<td>(b)</td>
</tr>
<tr>
<td>V</td>
<td>155</td>
<td>− + + + +</td>
<td>(c)</td>
</tr>
</tbody>
</table>

* Tentative assignment.
† ND, Not done.

Fig. 2(a to e) shows the curves obtained when antibodies 585, 322, 155, 173 and 298 respectively were iodinated and competed against the unlabelled antibodies. In almost every case, both homologous and heterologous antibodies, if they competed, were able to reduce binding of the labelled protein effectively to zero. Non-competing antibodies never showed significant differences from the negative control. In one case, however (173, Fig. 2d), the homologous antibody positive control was 100% effective, but the two positively competing molecules, 322 and 155, reduced binding by only 60 to 70%. This may indicate that the nature of competition is different in these instances and is discussed further below. In a second case (clone 298, Fig. 2e), the negative control curve itself dropped by 70%. This effect was entirely reproducible and was not improved after antibody was purified on a protein A–Sepharose column instead of by ammoni-
um sulphate precipitation. Conducting the assay at 4 °C or addition of labelled antibody before competitor similarly failed to improve binding. The basis of this effect is not known, but it is unlikely to be due to a non-specific bulk protein effect since all ascites fluids were diluted in PBS containing 1% BSA. Furthermore, this observation is not due to a low intrinsic avidity of antibody 298, since the unlabelled protein was just as active a competitor as the other ascites fluids (Fig. 2a to c, Fig. 3), and avidity as estimated from the RIA was comparable to that of other antibodies (Table 1). It seems likely, therefore, that the iodination procedure itself results in a significant detrimental effect on the function of this antibody leading to low-affinity binding and the high, non-specific competitive effect observed. Not all possible reciprocal competitions were performed with this antibody, because of the unacceptably high non-specific effects described above. However, provided positive competition had been observed it was not necessary to perform all the reverse experiments. Unlabelled antibody 298 was an effective competitor with all other labelled antibodies except 173 (Table 2), and the only information required was to determine whether unlabelled antibody 173 could compete with labelled antibody 298. In this experiment (Fig. 2e) competition by clone 173 was not greater than the negative control and we therefore tentatively assign antibodies 173 and 298 as non-competing.

A significant factor in these experiments is the role of avidity since a weakly binding antibody would not show detectable competition with a labelled, strongly binding immunoglobulin, but the reverse is clearly not true. When the low avidity antibody bears the label, strong competition should be evident. We therefore eliminated this effect by iodinating each antibody in turn, and performing reciprocal competition experiments. Thus, it is evident that antibody 173 does not compete with antibodies 322 or 155 when they carry the radioactive marker (Fig. 2b, c), but competition is clearly evident in the reverse experiment (Fig. 2d). The same procedure also avoids, by a similar argument, artefacts caused by great differences in specific antibody concentration.

The haemolysin-inhibiting monoclonal antibody

Virus-mediated haemolysis provides a convenient model system in which to study the effects of these antibodies on virus-induced cell fusion. The only H protein-directed monoclonal antibody which also possesses HLI activity so far isolated in our laboratory (antibody 26) was subjected to a similar analysis. After iodination this antibody retained a high binding capacity and behaved well in the competition RIA (Fig. 3). Antibody 26 behaved similarly to antibody 585, which expressed the same pattern of biological effects on H protein activity, but lacked fusion-inhibiting action. These two antibodies can now be placed in the same binding group. All antibodies except 173 were effective competitors with 26 and each was able to eliminate its binding completely.

From these data, it was apparent that the antibodies used fell into three binding groups. These are: those that exhibit positive competition with all other antibodies (clones 322 and 155); those that inhibit binding of all antibodies except 173 (clones 585, 298, and clone 26, which is also placed here by analogy to clone 585); those that inhibit binding of 322 and 155 only (clone 173). Radioiodinated antibodies 322 and 155 were not prevented from binding by unlabelled 173. However, competition was evident in the reverse experiment.

The binding groups are represented diagrammatically in Fig. 4. It is assumed that each binding group must represent an area on the H protein itself which can be larger than the combining site of any one antibody. The physical size and shape of these areas is not represented in Fig. 4, and no data are available on this point. Fig. 4 is thus a diagrammatic representation of the relationships between these groups and does not constitute a physical map.

DISCUSSION

The method applied in these experiments considers only competitive reduction in specific binding to be significant. The relative extent of competition was not taken into account. Unlabelled antibodies of known concentration and relative avidity were used as competitors. Iodination could conceivably decrease the avidity of the target antibody for its epitope, but should not lead to recognition of a different site. Consequently, such effects do not influence the
Measles haemagglutinin binding group classification described here. Furthermore, in order to interpret these data it was assumed that binding of one antibody would only inhibit another if the recognized epitopes overlapped, or were so close together that binding of one antibody sterically hindered access of the second to an adjacent epitope. This latter event may be uncommon and Stone & Nowinski (1980) have suggested that monoclonal antibodies may have considerable ‘swivel’ movement available when bound. Any possible allosteric inhibitory effects on binding have not been taken into consideration and such effects cannot be determined in these experiments. However, allosteric synergistic effects (Lubeck & Gerhard, 1982) were not observed using these antibodies. Each competing pair was examined by iodinating each antibody in turn, and in this manner relative avidity effects can be eliminated. This treatment would also serve to eliminate any variation in specific antibody titre within the ascites fluids.

We noted that one-way competition effects were identified in these experiments in the case of antibodies 173, 322 and 155, although differences in the avidities and concentrations of the unlabelled antibodies were small (Table 1). Avidity differences, however, might be accentuated by the iodination procedure and thus account for this observation. We found that the relative avidities and concentrations of all the untreated antibodies were very similar. Consequently, the differences in the titres of their biological effects (Table 1) can be assumed to reflect the functional importance of the areas of H protein recognized rather than variation in the intrinsic properties of the antibodies. In Fig. 4 it is apparent that antibodies of biological groups II and III were indistinguishable by competitive binding analysis and are now classed together in binding group 1. Groups IV and V were likewise similar and have been classed together in binding group 2. From Table 1 it is clear that while biological groups IV and V differ in the relative magnitudes of their HI and NT activities, titres of both were high compared with members of biological groups II and III. These more active antibodies have now been placed together in binding group 2, indicating that they recognize very closely apposed areas on the H protein. Thus, it is possible that they may be directed against a region of the H protein vital for its action. Antibody combination with adjacent areas (binding groups 1 and 3) is seemingly not so damaging to the functions of the molecule; combinations at the site recognized by antibody 173 have no biological effect at all.

The fact that tight binding of antibody to the virus attachment protein need not result in neutralization has now been observed in several viruses: mouse mammary tumour virus (Massey & Schochetman, 1981), murine leukaemia virus (Pinter et al., 1982) as well as measles virus (ter Meulen et al., 1981) and Sindbis virus (Chanas et al., 1982). The data presented here show that one such monoclonal antibody (298), which has a low HI action and lacks any neutralizing effect, binds similarly to other antibodies of generally low biological activity. This antibody is thus merely a special case in a set of monoclonal antibodies which display generally low biological activities. Antibodies with high NT and HI actions were also found similar in their binding pattern and distinguishable from the low-activity antibodies. Thus, although the relative magnitudes of the HI and NT titres of a given antibody may vary (Table 1; ter Meulen et al., 1981), in general the biological and binding patterns are associated. This supports the concept of an active region of the H polypeptide. Virus neutralization by the monoclonal antibodies examined here may therefore operate through the blocking of virus attachment, for which haemagglutination provides a model.

In most experiments, those competing antibodies assigned as positive were able to eliminate completely binding of the labelled protein. However, clones 322 and 155 were able to reduce the binding of labelled antibody 173 by only 60 to 70%. The significance of this is not known, but might indicate a different type of competition. Antibody 173 was unable to influence haemagglutinating activity or neutralize virus, suggesting that its binding site could be remote from those of the more biologically active antibodies. It is possible, therefore, that the overlap detected in this case is steric in origin rather than a true overlap of epitopes.

Interestingly, immunoglobulin 26, a monoclonal antibody with HLI activity, is completely excluded from its binding site on the H protein by all other preparations except 173. This strongly suggests that it must exert its HLI action from the H polypeptide and there is no additional binding site for antibody 26 on the F protein itself. Perhaps this H protein epitope is
close to the F protein on the surface of the virus, or alternatively this region of the H protein may be directly involved in the fusion process. Antibody 26 competes well with binding group 2 antibodies which lack a fusion-inhibiting activity. At the same time this antibody shows lower HI and NT activities. For this reason we suggest that the region of the H protein involved directly or indirectly in cell fusion is not contained in the area of overlap between groups 1 and 2 but must represent an area within binding group 1, uniquely recognized by antibody 26. This further suggests that areas of the H protein, here classified by the relationships between antibodies which bind to them, could certainly be dissected further. We are currently attempting to do this by selection of antigenic variants in the presence of monoclonal antibodies.

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REFERENCES


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