Immunoassays for Measles Virus Nucleocapsid Antigen: Effect of Antigen–Antibody Complexes

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SUMMARY

An inhibition radioimmunoassay and a two-site immunoradiometric (four-layer) assay for the quantification of measles virus nucleocapsid antigen were established and their sensitivities compared. Both assays exhibited threshold sensitivities of from 1 to 10 ng nucleocapsid antigen per ml. Concentrations of reagents used in the assays were shown to be an important factor determining the sensitivities of the assays. Purified mumps or parainfluenza 2 virus nucleocapsids did not compete with measles virus nucleocapsids in the inhibition assay but some degree of cross-reactivity with canine distemper virus nucleocapsid was observed. Pretreatment of virus-infected cells with detergent had a significant effect on the amount of antigen detectable by the assays. SDS greatly decreased the reactivity of measles virus nucleocapsid but Triton X-100 and, to a larger degree, sodium deoxycholate released antigen from the cells in quantities greater than that detected when no detergent pretreatment was employed. Complexing of antibodies to measles virus nucleocapsids in vitro decreased dramatically the threshold sensitivity of the nucleocapsid assays. The antigen–antibody complexes could be disrupted and the components separated but the conditions employed destroyed 90% of the antigenic reactivity of the nucleocapsid and also had a deleterious effect on the antibody. Nucleocapsid antigen was readily detected in brains from hamsters with acute measles encephalitis. The assays, however, were not sensitive enough for routine detection of antigen in brains from hamsters with chronic measles encephalitis.

INTRODUCTION

Measles virus can, at least on some rare occasions, cause long-term destructive diseases such as subacute sclerosing panencephalitis (SSPE) and possibly also multiple sclerosis (Adams & Imagawa, 1962; Connolly et al., 1967; Horta-Barbosa et al., 1969). Subacute and chronic central nervous system diseases have also been established in experimental animals by measles virus (Byington & Johnson, 1973). Infectious virus is not easy to demonstrate in these prolonged human or experimental infections of the central nervous system (Katz & Koprowski, 1973; Dubois-Dalcq et al., 1974; Johnson & Norrby, 1974). Although viral protein synthesis may be defective, leading to an incomplete assembly of virus particles, some viral antigens can be demonstrated in infected tissues. The detection of these antigens is usually done in histological sections using specific antisera and secondary anti-species sera tagged with peroxidase or fluorescing dyes (Johnson & Swoveland, 1977). The technique is mainly qualitative but very useful when well-characterized antisera such as monoclonal antibodies are available (Norrby et al., 1982). However, a more sensitive, quantitative assay would be needed in order to study these diseases in a more precise manner.

Sensitive and precise immunoassays for antibody measurements and antigen detection have been introduced in recent years (Hollinger et al., 1971; Rosenthal et al., 1972). Both radioactive tracers and enzymes can be used to label the components in these assays. Many factors are important when maximum sensitivity and simultaneous high reliability are needed. Such
sensitive immunoassays have recently been developed and used as tools for rapid virus diagnosis (Halonen & Meurman, 1982). If clinical specimens are taken early enough after disease onset, high success rates of specific diagnosis have been reached (Vesikari et al., 1981a).

Such sensitive assays may also be useful in studies of chronic viral infections in vitro as well as in human beings and in experimental animals (Forghani et al., 1978; Moore et al., 1978; Amesse & Payne, 1980; Vainionpää et al., 1982). Some attempts to use immunoassays in such studies have been made but the results have not always been promising. This may be due at least partially to the relatively insensitive techniques employed in some of those studies (Cook et al., 1981).

We have established different immunoassay techniques for measles virus nucleocapsid protein and have shown that about the same level of sensitivity is reached regardless of the immunoassay principle employed. Factors determining the threshold sensitivity, especially the effect of antibodies complexed to the antigen, have also been studied. The developed methods have been applied for detection of measles virus nucleocapsid in infected cells in vitro and in infected animals in vivo.

METHODS

**Cells and virus.** The wild-type strain of measles virus, isolated from a measles patient and subsequently single-plaque-cloned, was grown in monolayer cultures of Vero cells which were maintained using Eagle's basal medium supplemented with 10% foetal calf serum. Virus was purified from culture supernatants by the potassium tartrate gradient method described by Anttonen et al. (1980) and nucleocapsid from the infected cell pellet essentially according to the CsCl gradient method of Ilonen et al. (1979) but with the addition of a third gradient composed of 1.2 ml aliquots of 20%, 25%, 30%, 35% and 40% CsCl in phosphate-buffered saline (PBS). Following centrifugation at 4 °C in an SW27.1 rotor at 24000 r.p.m. for 16 h, the nucleocapsid bands were removed, diluted with PBS and pelleted in an SW27.1 rotor at 24000 r.p.m. for 1 h. The pellet was resuspended in PBS and used for immunization of animals and in the immunoassays. Nucleocapsids from canine distemper, mumps and parainfluenza 2 viruses were purified by the same procedure.

**Antisera.** Purified measles virus nucleocapsids in PBS were emulsified with an equal volume of Freund's incomplete adjuvant and injected intradermally into a rabbit and three guinea-pigs. This was followed at 3 week intervals by four identical injections, and finally the animals were exsanguinated. The rabbit received 5 × 100 μg of protein and each guinea-pig 5 × 50 μg of protein. The antisera were shown to be specific for the nucleocapsid polypeptide by immune precipitation.

Peroxidase-conjugated anti-rabbit IgG antibodies used in the enzyme immunoassay (EIA) were from Orion Diagnostica, Helsinki, Finland. Sheep anti-rabbit IgG antibodies used in the radioimmunoassays (RIA) were labelled with 125I using chloramidine-T (Ziola et al., 1978).

**Antibody radioimmunoassay.** Measles virus-infected cell lysate antigen for use in the assay was prepared as described by Lund & Salmi (1982). Solid-phase antigen was prepared by coating polystyrene balls (6.4 mm diam.; Precision Plastic Ball Co., Chicago, Ill., U.S.A.) with the antigen solution which was diluted with PBS to a protein concentration of 1 μg/ball. The polystyrene balls were also used as the solid phase for the antigen assays.

Samples to be tested for anti-measles antibody activities were serially diluted with PBS containing 0.5% bovine serum albumin, 0.5% Tween-20 and 0.02% sodium azide. Antigen-coated balls were added to duplicate 0.2 ml aliquots of each dilution and the samples incubated at 37 °C for 1 h. The balls were then washed twice with tap water and antibody bound to the balls quantified by incubation for 1 h at 37 °C with 125I-labelled anti-species antibodies.

**Measles virus-infected hamster brain material.** The Mantooth strain of measles virus was adapted to grow in hamster by six passages in newborn hamster brain. This virus was plaque-purified five times in Vero cells and passaged once more in newborn hamster brain. A 10% suspension of infected brain was made in PBS pH 7.4 by sonication. This seed virus was stored at −70 °C in small aliquots. Infectious virus could be isolated from these brains.

Newborn hamsters (age 5 to 7 days) were infected intracerebrally under ether anaesthesia with 20 to 25 μl of virus. The animals showed neurological signs of disease at 4 to 5 days and were moribund at 5 to 7 days after inoculation. The brains were harvested at this late stage of infection and stored at −70 °C until processed further.

Weanling hamsters (about 3 weeks old) were inoculated with 35 μl of virus under ether anaesthesia. These animals had some neurological signs at 1 to 2 weeks after inoculation. They deteriorated slowly and were moribund at 4 to 8 weeks after inoculation. The brains were removed at that stage and stored at −70 °C. Infectious measles virus could not be isolated from these animals.
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The infected hamster brains were placed in PBS such that the final concentration was 5% (w/v) and blended for 2 to 3 min on ice in a Sorvall Omnimixer. The blended suspensions were treated with 0.5% sodium deoxycholate (w/v) for 30 min and insoluble material removed by centrifugation at 600 g for 15 min. The supernatants were tested in the four-layer RIA after appropriate dilution with PBS containing 0.5% bovine serum albumin, 0.5% Tween-20 and 0.02% sodium azide.

RESULTS

Different techniques in measles virus nucleocapsid immunoassays

Two different immunoassay systems for nucleocapsid were established and shown to have sensitivities of between 1 and 10 ng antigen/ml, comparable to the sensitivity of a standard competitive RIA (Moore et al., 1978). In the two-site immunoradiometric (four-layer) assay, solid-phase guinea-pig immunoglobulins specific for measles virus nucleocapsid trapped the antigen which was then quantified by the secondary rabbit anti-nucleocapsid antibody followed by 125I-labelled anti-rabbit antibody. Optimal sensitivity of this assay was obtained within a relatively narrow concentration range of the guinea-pig immunoglobulin (Fig. 1). For routine use the solid phase was coated at a concentration of 360 ng of guinea-pig immunoglobulin per ball and the rabbit antiserum used in a dilution of 1/1000.

The second assay was an immunoassay inhibition method in which the sample competed with solid-phase antigen for a limited amount of rabbit nucleocapsid-specific antibodies (Vainionpää et al., 1982). The amount of antigen in the sample was inversely correlated to the amount of rabbit antibodies bound to the solid phase as quantified by 125I-labelled anti-rabbit antibodies (Fig. 2). The concentration of rabbit antiserum used in this assay was the critical variable and a dilution of 1/50000 gave the greatest combined reliability and sensitivity (data not shown).

Fig. 1. Effect of the primary 'catching' antibody concentration and the dilution of the secondary antibody on the results of the four-layer RIA. Polystyrene balls coated overnight at room temperature with the catching antibody were incubated at 37 °C for 3 h with a standard dilution of nucleocapsid antigen (100 ng/ml final concentration) diluted in PBS containing 0.5% bovine serum albumin, 0.5% Tween-20 and 0.02% sodium azide (RIA diluent). Following washing with water, the balls were incubated at 37 °C for 1 h with rabbit anti-nucleocapsid serum dilutions made in RIA diluent. Bound rabbit IgG was measured by incubation with a standard concentration of iodinated anti-rabbit IgG. The dilutions of the secondary rabbit anti-measles virus nucleocapsid antiserum were 1/1000 (●), 1/10000 ( ○) and 1/100000 (△).

Fig. 2. Specificity of the measles virus nucleocapsid assay. Purified nucleocapsids from cells infected with measles (●), distemper ( ○), mumps (△) and parainfluenza 2 (□) viruses were used in different concentrations in the inhibition type of assay.
Since the critical reagents for both of these assays were used in a highly diluted form, some day to day variation in sensitivity was observed but remained near the level of approximately 3 ng/ml. Enzyme immunoassay adaptations of these two techniques were equally reliable and sensitive and the resultant RIA and EIA curves essentially overlapped (data not shown).

Factors affecting the assay results

The specificity of the assay techniques was studied using purified nucleocapsids from distemper, mumps and parainfluenza 2 viruses. As Fig. 2 shows, the less-related mumps and parainfluenza 2 nucleocapsid antigens did not react at all in concentrations at least 10,000 times higher than measles virus nucleocapsid. Although distemper virus is relatively closely related to measles virus and there is clear cross-reactivity between their nucleocapsids (Örvell & Norrby, 1980), a more than 1000-fold greater reactivity was seen here in the homologous system.

Viral antigens can be released from infected cells by different treatments including detergents. Since this treatment of the nucleocapsid may have an effect on reactivity in the tests and may also expose hidden determinants in non-infected material, infected and non-infected Vero cells were treated with both non-ionic and anionic detergents. No reactivity was seen in the nucleocapsid tests with non-infected Vero cells when they were mechanically disrupted or treated with Triton X-100, SDS or sodium deoxycholate (data not shown). On the contrary, Triton X-100 and, to a greater extent, deoxycholate treatment exposed nucleocapsid material in infected cells (Fig. 3). The strongly anionic detergent SDS decreased dramatically the reactivity of the nucleocapsid material.

Use of the assay to detect measles virus nucleocapsid in vitro and in vivo

The sensitivity of the techniques was studied by measuring nucleocapsids in lytically infected cells. The minimum number of infected Vero cells releasing a detectable amount of nucleocapsid was approximately 100 when they were harvested at 72 h after infection with m.o.i. of 0.1 (data not shown). Harvesting at this time was found to yield the maximum amount of nucleocapsid material under our experimental conditions.

The four-layer antigen assay was also used to detect viral nucleocapsid antigen in brain material from hamsters with acute or chronic measles encephalitis. Nucleocapsid antigen was readily detected in brain tissue lysate derived from acutely infected hamsters, the amount varying from approximately 100 ng up to almost 700 ng per mg wet weight of whole brain (Table 1). In contrast, only rarely could nucleocapsid antigen be detected in brain tissue from chronically infected animals and in these cases the amount was less than 10 ng per mg of brain. In most assays of chronically infected brain material, binding values were not elevated above those obtained with control, uninfected hamster brain lysate (Table 1).

Effect of bound antibody on the assay results

Since antibodies produced during infection may complex to the corresponding antigen and influence the performance of the antigen assays, the effect of specific antibodies on these immunoassays was studied in vitro. Purified nucleocapsids and IgG isolated from a rabbit immunized with measles virus nucleocapsid were mixed to form immune complexes at different antigen–antibody ratios. The formed complexes were assayed for reactivity in a C1q immune complex assay (Arnadottir et al., 1982) and in the four-layer immunoradiometric assay for measles virus nucleocapsids. The results in Fig. 4 show that the sensitivity of the assay was rapidly lost when specific antibodies were bound to the nucleocapsid. The loss was about 100-fold when the complexes had reached maximum reactivity with C1q. Significant amounts of antigen remained undetected with vast antibody excess, shown at the left end of the curve, when the immune complex analysis also gave almost negative results.

Antigens would have to be released from possible immune complexes present in specimens from patients and from experimental animals in order to measure virus antigens efficiently. To establish such techniques we disrupted by low pH treatment the antibody–nucleocapsid complexes formed in vitro. After separation of the components in a Sephacryl S-300 column and subsequent neutralization, the fractions were tested for measles virus nucleocapsid and anti-
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**Fig. 3.** Effect of detergent treatment on the measles virus nucleocapsid antigen in lytically infected Vero cells. The cells in PBS were incubated with different detergents as noted below and dilution series of each lysate were tested for the antigen. ●, No detergent treatment; ○, 1% Triton X-100, 30 min on ice; △, 0.5% sodium deoxycholate, 30 min on ice; □, 0.1% SDS, 30 min on ice.

**Fig. 4.** Effect of increasing amounts of antibodies complexed to the antigen on the threshold sensitivity of the antigen assay (b). The binding of these complexes to solid phase C1q is also shown (a). Immune complexes were formed by addition of increasing amounts of purified measles virus nucleocapsid antigen to 1 μg aliquots of rabbit anti-nucleocapsid IgG, each sample being in a total final volume of 200 μl of 0.01 M-Tris-HCl pH 8.2, containing 0.07 M-NaCl, 1.5% Tween-20 and 0.1% sodium azide. Following overnight incubation at 4°C the C1q-binding immune complex assay was performed as described by Arnadottir et al. (1982).

**Table 1.** Detection of measles virus nucleocapsid antigen in acutely and chronically infected hamster brain by four-layer RIA

<table>
<thead>
<tr>
<th>Hamster brain</th>
<th>Nucleocapsid antigen (ng) per mg brain (wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic 1</td>
<td>3.6</td>
</tr>
<tr>
<td>Chronic 2</td>
<td>-*</td>
</tr>
<tr>
<td>Acute 1</td>
<td>648</td>
</tr>
<tr>
<td>Acute 2</td>
<td>162</td>
</tr>
<tr>
<td>Control</td>
<td>-*</td>
</tr>
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* Below detection limit.

nucleocapsid antibody activities. Results of one such experiment are shown in Fig. 5. Although separation of the antigen and antibody was not complete, both of these components of the immune complex could be separately identified in the fractions. The fact that the nucleocapsid was eluted from the column after IgG indicates that the nucleocapsid structure was disrupted to unit-size subunits by the harsh treatment employed.
Fig. 5. Separation of antibody and measles virus nucleocapsid immune complex components on a Sephacryl S-300 column equilibrated with 0.1 M-glycine-HCl pH 3.0. Immune complexes pre-formed in vitro (see legend to Fig. 4) were adjusted to pH 3.0 by dialysis against 0.1 M-glycine-HCl pH 3.0 for 3 h at 4 °C and then run through the column at a flow rate of 24 ml/h. Fractions of 0.4 ml were immediately neutralized and aliquots tested for measles virus nucleocapsid antigen by the four-layer RIA procedure (●) or for anti-measles nucleocapsid antibody activity by the procedure described in Methods (○).

Fig. 6. Effect of low pH treatment on anti-nucleocapsid antibody (a) and nucleocapsid antigen (b) activities. Antibody-binding activity was measured as described in Methods and antigen activity by the four-layer RIA procedure. ●, Control (untreated) assay; ○, stock solutions of antibody and antigen were pretreated with 0.1 M-glycine-HCl pH 3.0, for 3 h at 4 °C before being neutralized, diluted and tested in their respective assays. △, Stock solutions of antibody and antigen were treated as above, but at pH 2.4.

The calculated total amount of antigen recovered from the column was considerably less than the amount loaded. This was evidently due to inactivation of the antigen by the low pH treatment, which was confirmed by a separate experiment shown in Fig. 6(b). The measles virus nucleocapsid lost more than 90% of its antigenicity by the acid treatment which was also detrimental to the antibody activity (Fig. 6a). The separation and reactivity of antigen and antibody were similar after disruption of the complexes with 3.5 M-NaSCN (data not shown). The antigen and antibody components of the immune complexes could also be separated in low pH CsCl gradients but the separation and preservation of the antigen and antibody were not better than in the Sephacryl column chromatography procedure (data not shown).
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DISCUSSION

We have shown that measles virus nucleocapsid antigen can be detected at the same level of about 1 to 10 ng/ml using immunoassays based on different principles. Several factors, including design of the method itself, condition of the sample and specificity, titre and affinity of the antisera used have an effect on the test results. The sensitivity of the assay is of prime importance when small amounts of antigen are to be detected, as in rapid virus diagnosis (Halonen & Meurman, 1982) or in studies of defective virus infections in vivo (Johnson & Swoveland, 1977). Antibodies complexing to the antigen can especially reduce the sensitivity of the test or totally block detection of antigen in these immunoassays.

Optimizing of the amounts of reagents was found to be an important factor when maximum assay sensitivity was aimed for. The general threshold sensitivity of this type of assay, between 1 and 10 ng protein/ml, seems to be sufficient for detection of antigens in clinical specimens during viral infections (Vesikari et al., 1981b). Because material from a minimum of about 100 infected cells is needed to reach the detection threshold, such conditions should be easily met during the acute phase of most viral infections when the antibody response is only beginning to appear.

These antigen assays were primarily established in order to detect antigens in vivo in encephalitis of experimental animals. Nucleocapsid antigen was readily detected in brain tissue from acutely infected animals but in chronically infected animals antigen levels were just at or below the detection threshold. This was not unexpected because only a minority of the brain cells appears to be infected in such infections in vivo (Johnson & Swoveland, 1977). It is not certain that the amount of nucleocapsids produced per cell in the central nervous system is the same as in lytically infected Vero cells in vitro. We have shown earlier that hamster brain cells persistently infected in vitro produce only about 3% of the measles virus nucleocapsid at 37 °C (Vainionpää et al., 1982) as compared to the amounts produced in vitro in this work. For these reasons it is understandable that the sensitivity of the present immunoassay techniques may not be high enough for studies of chronic and defective measles virus infection in the central nervous system.

One very important factor in antigen immunoassays during viral infections in human beings and in experimental animals is the simultaneous presence of specific antibodies. Circulating immune complexes seem to be a very common phenomenon during acute viral infections (Charlesworth et al., 1978; Nielsen et al., 1980; Ziola et al., 1983). At least a part of these complexes seem to consist of viral antigens and specific antibodies (Coyle et al., 1982; Ziola et al., 1983). Such complexes have also been shown to form in vitro when virus-infected cells are incubated with specific antibodies (Perrin & Oldstone, 1977). As shown in this work, such complexes may still be detectable in antigen immunoassays if they are formed in antigen excess. Such a situation may exist for only a brief time during the early phase of antibody synthesis. Ultimately, antibody excess will be reached and the antigen assay will rapidly lose its sensitivity. Even immune complex assays may give negative results although complexes in antibody excess are abundant in the circulation. Such complexes in antibody excess may at least partially explain the relatively rapid disappearance from excretions of virus antigens detectable by immunoassays (Vesikari et al., 1981a).

The complexing of specific antibodies to the antigen may also partially explain the poor results of antigen detection in the chronically infected animal experiments. It has been shown that antibodies can complex in situ to their corresponding antigen present in tissue (Johnson & Swoveland, 1977). Measles virus antigen in brains of SSPE patients has also been shown to be masked by antibodies (Jenis et al., 1973). Such a phenomenon may be one reason for the detection of measles virus antigen on only a few cells in chronically infected animals when the cells are stained with specific antiserum for fluorescent microscope examination (Johnson & Swoveland, 1977).

Since antibodies complexed to antigens in specimens reduce the sensitivity of the antigen immunoassays, the complexes should be broken and the components separated. Such methods were devised in this study. However, although the antigen and antibody could be separated, the harsh treatment required destroyed most of the antigenic activity and also reduced the binding
capacity of the antibody. This inactivation did not cause problems if the amount of antigen in the samples treated was high enough, but it was very critical when only small amounts of antigen were to be measured. This inactivation during treatment may be reduced if the separation time can be decreased. It may also be possible to develop more convenient methods which can efficiently detect antigens under conditions of antibody excess if high affinity monoclonal antibodies are available (Schafritz et al., 1982). Such methods, which do not require any prior disruption of the immune complexes, may be the most practical ones if a larger number of specimens are to be tested on a routine basis.

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