

PRODUCTION AND PROPERTIES OF MONOCLONAL ANTIBODIES AGAINST AFRICAN HORSESICKNESS VIRUS, SEROTYPE 3

W. VAN WYNGAARDT^{(1)*}, D. H. DU PLESSIS⁽¹⁾, SANDRA VAN WYNGAARDT⁽²⁾ and J. A. VERSCHOOR⁽²⁾

ABSTRACT

VAN WYNGAARDT, W., DU PLESSIS, D. H., VAN WYNGAARDT, SANDRA & VERSCHOOR J. A., 1992. Production and properties of monoclonal antibodies against African horsesickness virus serotype 3. *Onderstepoort Journal of Veterinary Research*, 59, 129–133 (1992)

Four polyethylene glycol-mediated cell fusions yielded a total of 23 monoclonal antibodies (McAbs) specific for African horsesickness virus (AHSV). Two recognised the major core structural polypeptide, VP7, while one each was specific for the outer capsid proteins, VP2 and VP5. The remainder co-precipitated both VP2 and VP7. An inhibition ELISA and radio-immunoprecipitation revealed two types of co-precipitating McAbs, distinguishable from each other by the different relative amounts of the two proteins they precipitated. Only co-precipitating McAbs reduced the size and number of plaques formed by AHSV on VERO cell monolayers, but even at low dilution did not completely abolish virus infectivity. A McAb specific for VP7 showed potential as a group-reactive diagnostic reagent since guinea pig antisera to all nine serotypes of AHSV, as well as an anti-serotype 4 horse serum and an anti-serotype 3 rabbit serum, inhibited its binding in ELISA to AHSV serotype 3.

INTRODUCTION

African horsesickness virus (AHSV), an orbivirus that infects equidae, causes a severe, often fatal disease of considerable economic importance. The AHSV group embraces nine serotypes which complicates vaccination and routine diagnosis and are distinguished from each other by their neutralization behaviour (McIntosh, 1958; Howell, 1962). The proteins and nucleic acids of AHSV have been characterized (Bremer, 1976; Bremer, Huismans & Van Dijk, 1990) and rapid immunodiagnostic assays for both the antigen and virus-specific antibodies have been reported (Williams, 1987; House, Miki-ciuk & Berninger, 1990; Du Plessis, Van Wyngaardt & Bremer, 1990; Du Plessis, Van Wyngaardt, Gerdes & Opperman, 1991; Hamblin, Mertens, Mellor, Burroughs & Crowther, 1991). It has long been established that AHSV does not cross-react with other orbiviruses in the complement fixation test (Erasmus, Boshoff & Pieterse, 1978).

By comparison, the antigenicity of the prototype orbivirus, bluetongue virus (BTV), has been extensively investigated. In these studies, monoclonal antibodies (McAbs) have played a central role. It was confirmed for instance, that VP2, one of the two outer capsid polypeptides, possesses epitopes that are recognised by neutralizing McAbs (Gould, Hyatt & Eaton, 1988; Ristow, Leendersten, Gorham & Yilma, 1988; Heidner, Rossitto & Maclachlan, 1990) and that regions of VP7, the major inner polypeptide, are accessible on the virus surface (Hyatt & Eaton, 1988; Lewis & Grubman, 1990; Eaton, Gould, Hyatt, Coupar, Martyn & White, 1991). Moreover, BTV diagnosis has been facilitated by the availability of a competitive ELISA utilizing a sero-group-specific McAb (Afshar, Thomas, Wright, Shapiro & Anderson, 1989).

This report concerns the production and characterisation of AHSV-specific McAbs. Specificities for the structural polypeptides of the virus were determined in immunoprecipitation assays and inhibition of viral plaque formation was examined. In addition, the ability of a VP7-specific McAb to detect antibodies to all nine AHSV serotypes in an inhibition ELISA is described.

MATERIALS AND METHODS

Propagation and purification of AHSV

Inocula of AHSV serotype 3 were obtained from Dr B. J. Erasmus of the World Reference Centre for AHSV, Onderstepoort. The virus was propagated in baby hamster kidney cells and purified as described for bluetongue virus (Huismans, Van der Walt, Cloete & Erasmus, 1987).

Production of McAbs

Mice (BALB/c or C57BL/6) were initially immunized with approximately 30 µg of purified AHSV in low Tris buffer (LTB, 2 mM Tris, pH 8.6), emulsified in complete Freund's adjuvant and boosted subcutaneously with the same amount of virus in complete Freund's adjuvant about 2 weeks later. Final boosters in LTB were administered intraperitoneally after a further 3–5 weeks. Three days after the final injection the mice were sacrificed to obtain splenocytes for fusion. Splenocytes from immunised BALB/c or C57BL/6 mice were fused with Sp2/0 myeloma cells using polyethylene glycol (PEG) (Galfré & Milstein, 1981). Supernatant fluids from hybridoma cultures were screened by indirect ELISA.

Antisera

Guinea pig antisera to the AHSV serotypes were provided by the World Reference Centre, Onderstepoort. Horse antiserum was obtained from an animal that had recovered from experimental infection with AHSV serotype 4. Rabbit serum was obtained from an animal immunized with purified AHSV serotype 3.

ELISA

Screening assay for McAbs: Indirect ELISA was performed essentially as described by Engvall

* Author to whom correspondence should be addressed

⁽¹⁾ Biochemistry Section, Veterinary Research Institute, Onderstepoort, 0110, Republic of South Africa

Fax No: South Africa (012556573)

Tel. No: (012) 5299227.

⁽²⁾ Department of Biochemistry, University of Pretoria, Pretoria 0001

(1980). Polystyrene microtitre plates (Nunc, Denmark) were coated with purified AHSV (100 µl/well) at a concentration of 20 µg/ml in LTB. For blocking, the wells were incubated for 1 h at 25 °C with 10 % v/v foetal calf serum in PBS which was also used for all antiserum and ascitic fluid dilutions. For screening, hybridoma supernatant fluids were tested undiluted. The isotypes of the McAbs were determined by indirect ELISA with antisera specific for mouse heavy chains (Cappel, Denmark). The substrate used was o-phenylenediamine and colour development was monitored at 450 nm.

Inhibition ELISA: The method of Lunt, White & Blacksell (1988) was used but with the diluent reported above. Guinea pig, rabbit, and horse antisera were diluted 1/100 to test their ability to inhibit the binding of McAb 1F1 to purified AHSV serotype 3. Ascitic fluid of McAb 1F1 was diluted 1/200 when guinea pig and rabbit anti-sera were assayed and 1/400 when the horse serum against AHSV serotype 4 was tested. In experiments using biotinylated antibody, the blocking solution was 5 % (w/v) non-fat milk powder in phosphate buffered saline (PBS) and antibody dilutions were made in PBS containing 0,5 % (w/v) milk powder and 0,05 % (v/v) Tween 20.

Determination of polypeptide specificity of McAbs

³⁵S-labelled cell lysates were prepared according to Huismans *et al.* (1987). Polypeptide-antibody complexes were precipitated by a *Staphylococcus aureus* bacterial adsorbent (Bio-Yeda, Israel) and analysed by polyacrylamide gel electrophoresis and autoradiography (Blanton & Tevethia, 1981; Kessler, 1975).

Biotinylation of McAbs

The IgG fraction from ascitic fluid was obtained by ammonium sulphate precipitation. After dialysis against 0,1 M sodium carbonate buffer pH 8,3, 25 µl of 2 mg/ml biotinamidocaproate N-hydroxysuccinimide ester (Sigma) in dimethyl formamide was added to the dialysate. After 2 h incubation at room temperature, unreacted ester was removed by exhaustive dialysis against PBS (Harlow & Lane, 1988).

Plaque reduction and inhibition assays

An AHSV inoculum was diluted with LTB to contain 2,6 × 10³ PFU/ml. Equal volumes of test sera or control samples were added and the mixture was incubated at 20 °C for 40 min before assay on VERO cell monolayers (Hopkins, Hazrati & Ozawa, 1966; Oellermann, 1970).

RESULTS

Specificity of McAbs for AHSV polypeptides

Using spleen cells from immunized mice, four separate PEG-mediated cell fusions produced 23 stable hybridomas secreting McAbs that recognised purified AHSV in an indirect ELISA. The specificities of all the McAbs for the individual virus structural proteins were determined by immunoprecipitation from a radiolabelled AHSV-infected cell lysate. Immunoprecipitation showed that one McAb recognised VP5 (Fig. 1, McAb 4F2, lane a), two were

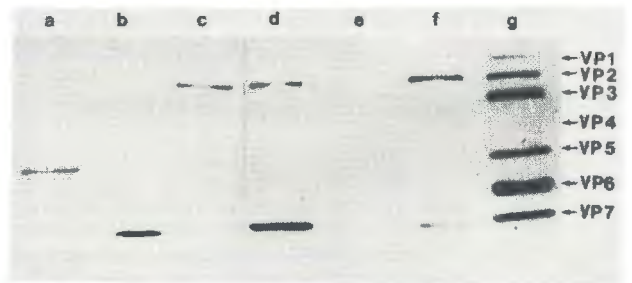


FIG. 1 Composite autoradiograph showing polypeptides precipitated from a radiolabelled AHSV-infected cell lysate by: (lane a) McAb 4F2; (lane b) McAb 1F1; (lane c) McAb 4F4; (lane d) McAb 4F1; (lane e) normal mouse serum; (lane f) McAb 2F2; (lane g) mouse antiserum against AHSV.

specific for VP7 (e.g. McAb 1F1, lane b) and one for VP2 (McAb 4F4, lane c). The remaining 19 co-precipitated two of the major proteins, namely VP2 and VP7. Two of these precipitated less VP2 compared to VP7 (e.g. McAb 4F1, lane d), while the remainder (e.g. McAb 2F2, lane f) precipitated more VP2 than VP7. The isotypes, polypeptide specificity and nomenclature of the five representative McAbs that were further characterized are summarized in Table 1.

TABLE 1 Characteristics of representative monoclonal antibodies directed against AHSV serotype 3

Designation	Isotype	Specificity
4F2	IgG2b	VP5
1F1	IgG2b	VP7
4F4	IgM	VP2
2F2	IgG1	VP2+VP7
4F1	IgG2a	VP7+VP2

Since the co-precipitation of two different polypeptides by a single McAb need not necessarily mean that the antibody recognizes both (Lee, Hayes & Joklik, 1981a), the specificity of one of the co-precipitating antibodies, 2F2, was further examined. The McAb 1F1, which was specific for VP7 alone (Fig. 2, lane a), was used to remove all detectable VP7 from a ³⁵S-labelled cell lysate. The remaining polypeptides were then treated with McAb 2F2 to determine whether it recognised VP2 in the absence of VP7. Fig. 2, lane b, shows that VP2 was indeed precipitated from the VP7-depleted lysate. The removal of VP7 was confirmed since this protein was not precipitated from the resulting supernatant fraction with a mouse antiserum to AHSV virions (lane c). Although no visible VP2 band was observed in lane c, a low titre of VP2-specific antibodies measured in the mouse serum used as a positive control suggested the presence, albeit at a low concentration, of anti-VP2 antibodies. This mouse antiserum was that which was used to obtain the result depicted in lane f. In this lane, the VP2 band is barely visible but the polyclonal guinea pig serum used as an additional positive control (lane e) yielded a clearly discernible

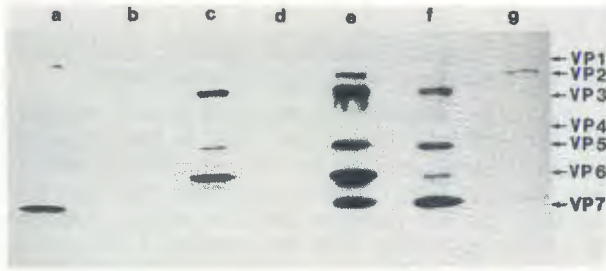


FIG. 2 Determination of the polypeptide specificity of the co-precipitating McAb 2F2. Autoradiograph showing precipitation of radiolabelled AHSV polypeptides. (Lane a) polypeptide VP7 precipitated from ³⁵S-labelled total cell lysate by McAb 1F2; (lane b) supernatant fluid from (a) incubated with McAb 2F2 showing precipitation of VP2 from cell lysate depleted of VP7; (lane c) supernatant fraction from (b) incubated with an anti-AHSV mouse serum showing absence of VP7; (lane d) total lysate incubated with normal mouse serum; (lane e) guinea pig AHSV antiserum; (lane f) mouse AHSV antiserum; (lane g) reaction of McAb 2F2 with undepleted cell lysate showing co-precipitation of VP2 and VP7.

VP2 band. This indicated that the original cell lysate initially contained both VP2 and VP7. Pre-immune mouse serum did not precipitate any proteins from the lysate (lane d).

Using a biotinylated McAb, an attempt was made to establish whether examples of the two apparently different categories of co-precipitating antibody were directed against the same antigenic region. McAb 2F2, which manifested a more intense VP2 than VP7 band on the autoradiograph (Fig. 1, lane f), was labelled with biotin and tested in an inhibition ELISA to establish whether its binding could be inhibited by a McAb that produced the opposite VP2/VP7 co-precipitation pattern. A McAb (3F2)

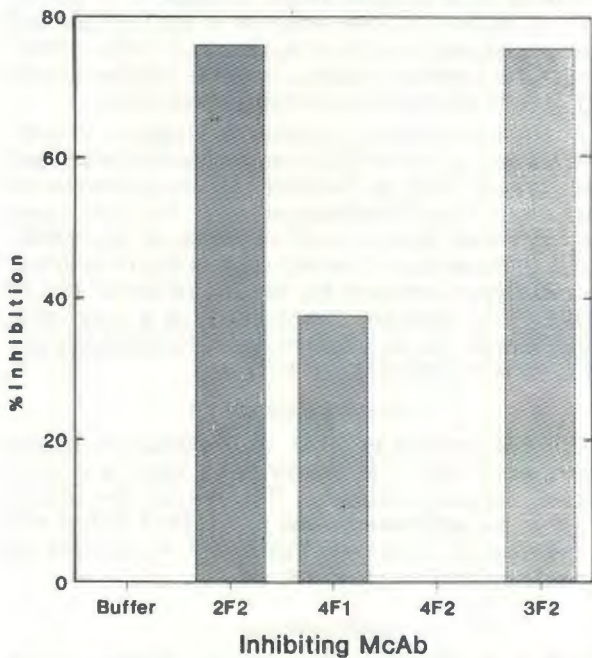


FIG. 3 Inhibition of biotinylated co-precipitating McAb 2F2 by unlabelled McAbs in an inhibition ELISA. Competing antibody was used at a dilution of 1/100.



FIG. 4 Plaque reduction and neutralization activity of McAb 2F2. AHSV incubated with (a) LTB and (b) an equivalent volume of McAb 2F2 at a dilution of 1/25, prior to assay on confluent VERO cell monolayers.

with the identical VP2/VP7 precipitation ratio (not shown), reduced the ELISA signal by almost 80 %, but McAb 4F1 which showed the converse pattern (Fig. 1, lane d) was capable of less than 40 % inhibition (Fig. 3). This finding suggests that the two types of co-precipitating McAb may have recognized the same, or closely adjacent epitopes. A McAb (4F2) directed against an epitope on VP5 had no inhibitory ability while unlabelled McAb 2F2 inhibited its labelled counterpart by almost 80 %.

Plaque reduction and inhibition assays

Of the McAbs listed in Table 1, only the co-precipitating McAbs 2F2 and 4F1 inhibited the plaque-forming ability of AHSV on VERO cell monolayers. Neither, however, completely abolished infectivity, even at ascitic fluid dilutions of 1/5. Both partially inhibited plaque formation. This was manifested by a reduction in the number of plaques by approximately 40 % after incubation of the AHSV inoculum with 25 × diluted ascitic fluid. Furthermore, the average size of the remaining plaques was decreased compared to the control samples which had been treated with either non-immune serum or LTB. Fig. 4 shows the effect of McAb 2F2 on AHSV infectivity.

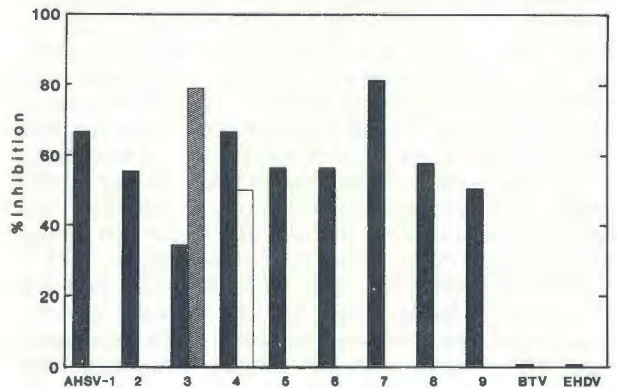


FIG. 5 Inhibition of the binding of the VP7-specific McAb 1F1 by antisera to the nine AHSV serotypes and two other orbiviruses, bluetongue virus (BTV) and epizootic haemorrhagic disease virus (EHDV), in an inhibition ELISA. Antisera raised in: guinea pigs (solid black); rabbit (diagonal lines); horse (white). The horse serum was raised against AHSV serotype 4 and the rabbit serum was raised against AHSV serotype 3.

Serogroup reactivity in an inhibition ELISA

A potential application of McAbs is in diagnostic immunoassays for the African horsesickness serogroup. McAb 1F1 was previously evaluated as a detecting antibody in a F(ab')₂ ELISA but was found to be unsuitable for that purpose (Du Plessis *et al.*, 1990). However, in an inhibition ELISA, individual guinea pig antisera directed against all nine AHSV serotypes inhibited the binding of this VP7-specific McAb (1F1) to the virus with varying degrees of efficiency (Fig. 5). At the same dilution the horse serum against AHSV serotype 4 and the rabbit serum against AHSV serotype 3 reduced the binding of McAb 1F1 to purified AHSV serotype 3 by 50 % and 80 % respectively. The inhibition of 33 % obtained with the homologous serotype 3 guinea pig antiserum (Fig. 5) could perhaps be attributed to low antibody titre or avidity in this particular serum. Antisera against two other orbiviruses, namely BTV serotype 4 and epizootic haemorrhagic disease virus (EHDV), had no significant effect.

DISCUSSION

A peculiarity of the AHSV McAbs produced was that only four recognised individual viral polypeptides, while 19 co-precipitated the outer capsid protein, VP2, together with VP7 which is part of the virus core. The large proportion of co-precipitating McAbs in the panel may be an indication that the relevant epitopes are intrinsically immunodominant, or alternatively, that during selection of AHSV-reactive McAbs by indirect ELISA, the particular assay format favoured the binding of these antibodies. It is known that proteins may be distorted by attachment to a plastic surface (Friguet, Djavadi-Ohanine & Goldberg, 1984; McCullough Crowther & Butcher, 1985; Butler, Spradling, Suter, Dierks, Heyermann & Peterman, 1986; Dierks, Butler & Richerson, 1986) and this could conceivably have exposed the epitope or epitopes responsible for co-precipitation.

The simultaneous precipitation of two reoviral proteins by a single McAb is not without precedent and has been described for both BTV (Appleton & Letchworth, 1983) and reovirus (Lee *et al.*, 1981a). In the case of reovirus, it was attributed to the formation of a complex between the proteins μ 1 and μ 1C, with the antibody recognizing an epitope on one of the two proteins. It seems improbable, however, that the two AHSV proteins were in the form of a complex in our cell lysate, since the co-precipitating AHSV McAb (2F2) recognized VP2 in a lysate that had been depleted of VP7. If McAb 2F2 did in fact recognize a unique epitope that is situated only on VP2 in a VP2-VP7 complex, our findings imply that the VP7-specific McAb used for the removal of VP7 dissociated the putative complex. This has been reported to occur with the Sindbis virus envelope proteins E1 and PE2 (Clegg, Chanas & Gould, 1983). Another possibility is that the co-precipitating AHSV VP2-VP7 McAbs recognized cross-reacting epitopes on VP2 and VP7. Two categories of co-precipitating McAbs could be distinguished by the ratio of the intensity of the VP2 band compared to VP7 on the autoradiograph. These differences could conceivably reflect different relative affinities for two separate epitopes, one on each polypeptide.

It appears unlikely though, that two viral proteins, each with a distinct structural function, would have strongly cross-reacting antigenic determinants.

The partial inhibition of one type of co-precipitating VP2-VP7 McAb by the other in the inhibition ELISA suggests that the two co-precipitating antibodies were directed against epitopes that are not identical, but are perhaps neighbouring or overlapping. The possibility that the paratopes recognize discontinuous epitopes resulting from the juxtaposition of amino acid residues on both polypeptides (Van Regenmortel, 1985) therefore cannot be excluded. Since McAb 2F2 precipitated VP2 in the absence of VP7, it would have had to retain residual affinity for a portion of the epitope on the remaining protein. This last explanation would imply an intimate association of antigenically accessible regions of the inner and outer proteins in the virion.

Two of the McAbs (2F2 and 4F1) had an inhibitory effect on AHSV infectivity, but because both were VP2-VP7 co-precipitators, it was impossible to unequivocally determine whether their effect was due to specificity for epitopes on either VP2 or VP7. By analogy with the structurally related BTV, it may be postulated that the corresponding AHSV outer polypeptide, VP2, is a similar target in antibody-mediated virus neutralisation (Appleton & Letchworth, 1983; Huismans *et al.*, 1987; Gould *et al.*, 1988; Ristow *et al.*, 1988; Heidner *et al.*, 1990;), but our only VP2-specific AHSV McAb, an IgM (4F4), had no effect on infectivity.

Two mechanisms responsible for the neutralization of infectivity by antibodies are the blocking of viral penetration into host cells (Fuller, Santos & Spear, 1989) and prevention of viral attachment (Lee, Hayes & Joklik, 1981b). We did not obtain complete neutralization, even at dilutions of ascitic fluid as low as 1/5. The reduction in the number and size of plaques may thus be due to a partial inhibition of the infective process, with the McAbs retarding, but not efficiently preventing viral ingress.

In addition to their potential applications in antigenic analysis, the AHSV-specific McAbs described here should help to facilitate the development of diagnostic enzyme-immunoassays. This has been the case with McAbs to BTV (Afshar *et al.*, 1989). Since immune sera directed against the nine different serotypes inhibited the binding of McAb 1F1 to purified viral antigen, this antibody is a promising candidate for use in a routine assay for antibodies to all members of the AHSV serogroup.

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