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Blocking measles virus infection with a recombinant soluble form of, or monoclonal antibodies against, membrane cofactor protein of complement (CD46)

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SUMMARY

Human membrane cofactor protein (MCP, CD46) functions as an inhibitor of the complement (C) cascade to protect host cells from C attack, and as a receptor for measles virus (MV). Normal human sera contains 10–60 ng/ml of naturally produced soluble forms of MCP, which is also a cofactor for the factor I-mediated inactivation of C3b. We produced monoclonal antibodies (mAb) against MCP and a recombinant soluble form of MCP similar to the natural soluble forms, and tested their ability to block MV infection. Vero cells and CHO cells expressing human MCP were the targets. Of the antibodies tested, M75 and M177, which blocked the C regulatory activity of MCP, efficiently blocked MV infection. More than 50 μg/ml of the soluble form moderately blocked MV infection of CHO cells expressing MCP, but barely blocked that of Vero cells. The two mAb and the soluble form also inhibited MV H protein-mediated green monkey erythrocyte rosette formation. A quantitative analysis suggested that 30 μg/ml of the soluble form functionally corresponded to 0·2 μg/ml of M177 or M75. These data established that the C regulatory function and the MV receptor function of MCP were blocked simultaneously by the individual mAb, and that soluble forms of MCP could inhibit MV infection in cells expressing human MCP, although doses far higher than the natural concentration of soluble MCP were required.

INTRODUCTION

Membrane cofactor protein (MCP; CD46) was first purified and characterized as a membrane complement (C) regulatory protein.[1] It serves as a cofactor for plasma protease factor I, and facilitates the inactivation of deposited C3b to C3bi.[1,2] Its primary role is to protect host cells in conjunction with decay-accelerating factor (DAF)[2–4] from homologous C attack[3,5,6] by blocking activation of the C cascade. All nucleated cells reported to date possess MCP even if they lack DAF,[7,8] suggesting that it is important for cell survival in an environment where C can be activated.

MCP consists of the N-terminus of four short consensus repeats (SCR), a serine/threonine-rich (ST) region, a sequence of unknown significance (UK), a transmembrane domain (TM), and a 16 or 23 amino acid cytoplasmic tail (CYT).[9] SCR2, 3 and 4 are responsible for C regulation (Fig. 1). A characteriztic feature of MCP is the use of alternative splicing to produce four commonly expressed forms that are named ST^C and ST^{BC} with short (CYT1) and long (CYT2) tails,[10] namely ST^C/CYT1, ST^C/CYT2, ST^{BC}/CYT1, and ST^{BC}/CYT2, which cause the genetic and organ-specific heterogeneity of MCP.[11–14] Although soluble forms of MCP are present in some body fluids,[15,16] their structures, functions and roles are not yet defined.

Recently, MCP was found to be a measles virus (MV) receptor.[17,18] Its wide distribution is consistent with the variety of signs observed during MV infection. The MV binding site on MCP is mapped on SCR1 and SCR2 (Fig. 1): MCP mutants

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Abbreviations: C, complement; CR1, C3b/C4b receptor (CD35); DACM, *N*-(dimethylamino-4-methylcoumarinyl)maleimide; DAF, decay-accelerating factor (CD55); DMEM, Dulbecco's minimal essential medium; DPBS, Dulbecco's phosphate-buffered saline; f-C3(MA), methylamine-treated fluorescent-labelled C3, which is a substrate for factor I; GME, green monkey erythrocytes; GVB, gelatin veronal buffer; mAb, monoclonal antibody; MCP, membrane cofactor protein (CD46), recombinant soluble forms are indicated as γMCP; MV, measles virus; PBS, phosphate-buffered saline; PFU, plaque-forming unit; SCR, short consensus repeats; ST, the serine/threonine-rich domain in MCP, consisting of three domains named ST^A, ST^B, and ST^C.

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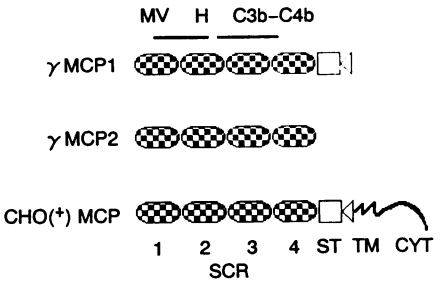

Figure 1. Scheme of recombinant soluble and membrane MCP. Recombinant soluble MCP (γMCP1 and γMCP2, see the text) and CHO^+ cell membrane MCP, which is the ST^C/CYT2 phenotype,[11,12] are shown. The overlines indicate a putative MV H protein binding site and a C3b–C4b binding region. The SCR (1–4), the serine/threonine-rich domain (ST), the unknown significance (indicated by open triangles), the transmembrane domain (TM), and the cytoplasmic tail (CYT) are indicated.

lacking SCR1 or SCR2 lose MV binding ability.[19] We and others have produced monoclonal antibodies (mAb) against MCP,[7,20–22] the epitopes of which are different.[19] We have also produced recombinant soluble forms of MCP, and their inhibitory effect on the C regulatory function of MCP has been investigated.[23,24] However, the ability of these reagents to block MV infection has not been characterized.

Here, we show that two mAb against MCP, both capable of inhibiting cofactor activity of MCP, and a soluble form of MCP, which has C regulatory activity, act as inhibitors of MV infection.

MATERIALS AND METHODS

Reagents and cells

Wild-type CHO cells were obtained from the American Type Culture Collection (ATCC Bethesda, MD). They were maintained in coated dishes (Nunc, Roskilde, Denmark) in Ham's F12 (Nissui Co., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS). Cultures were maintained in a 5% CO_2/95% air atmosphere at 37°. The green monkey kidney line, Vero cells and green monkey erythrocytes (GME) were from the Research Institute for Microbial Diseases (Osaka University, Japan). Vero cells were cultured in minimal essential medium (MEM)/5% FCS.

Monoclonal antibodies against MCP (M75, M160 and M177) were produced and purified in our laboratory as described elsewhere.[7] Other mAb, MH61[20] and E4.3,[21] were gifts from Drs M. Okabe (Osaka University, Japan) and B. Loveland (Austin Institute, Australia), respectively, and those named J48 and S19S were from Dr J. Pesando (Onco membrane Inc., Seattle, WA, USA).[23] The epitopes of these mAb were mapped[19,25] and the results are summarized in Table 1. Monospecific antibodies against ST^A and ST^C were produced by injecting the peptides coding ST^A and ST^C into rabbits by the method of Semba *et al.*[26] A mAb against human CR1 (243R), which recognizes green monkey CR1 (data not shown), was established in our laboratory and its properties have been described elsewhere.[8] A mAb against C3bi (G3E)[27] was kindly donated from Dr K. Iida (Takara Co., Kyoto, Japan). Fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG was a product of Cappel (Treyburn, NC). A mAb against MV H protein, which has haemagglutination inhibitory activity and MV neutralizing activity, was produced by Dr S. Ueda (Osaka University, Japan).

Table 1. Mean fluorescence shifts by mAbs against MCP

mAb	Epitope	Vero	GME	CHO^+
Mouse IgG	—	0·606	0·497	0·823
J48	SCR1	0·892	0·865	18·69
S19S	SCR1	0·631	0·604	18·02
E4.3	SCR1	0·634	3·752	18·80
M75	SCR2	34·43	23·88	26·12
M177	SCR2	36·36	24·26	25·94
MH61	SCR3	10·26	ND	6·605
M160	SCR3	5·683	2·303	19·76

ND, not determined.

Human C3[28] and factor I[29] were purified as described elsewhere. MV was a Nagahata strain that had undergone four passages in hamster brain.[30,31] *N*-(dimethylamino-4-methyl-coumarinyl)maleimide (DACM) was purchased from Wako Pure Chemical Co. (Tokyo, Japan). Trypsin and kanamycin were from Sigma Co. (St Louis, MO). Restriction enzymes were from Takara Biomedicals (Kyoto, Japan).

Preparation of CHO cells expressing MCP

Plasmid construction and transfection methodology were as described elsewhere.[32] Briefly, a full-length cDNA of MCP (ST^C/CYT2 phenotype)[9] was subcloned into the *Eco*R1/*Pst*1 site of the pME18S. The vector was transformed into *Escherichia coli* C600 (Toyobo Inc., Osaka, Japan) and constructs containing the insert in the sense (+) and anti-sense (−) orientations were isolated, characterized, and amplified by standard means.[33] About 5×10^5 CHO cells/tissue culture plate were cotransfected with 20 μg of pME–MCP and 1 μg of pSV2hph[34] by means of calcium phosphate precipitation.[33] Transfected CHO cells were maintained in Ham's F12 medium, 10% FCS, 0·06% kanamycin, in an atmosphere of humidified 5% CO_2/95% air. Hygromycin-resistant colonies were isolated with cloning cylinders and expanded in tissue culture plates and flasks. Phosphate-buffered saline (PBS) containing 0·02% (w/v) EDTA and 0·005% trypsin was used to detach the cells. This concentration of trypsin did not alter MCP on CHO cells.[6] The expression of MCP was confirmed by flow cytometry. CHO cells expressing MCP (CHO^+ cells) and those containing anti-sense cDNA (thus expressing no MCP; CHO^- cells) were established.

Recombinant soluble forms, γMCP1 (279 amino acids) and γMCP2 (251 amino acids), were also produced by a similar method (Fig. 1). These forms were harvested from culture supernatants and immunoaffinity purified.[35] The purity of the proteins were confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).[36] The structure of γMCP1 was confirmed by DNA sequencing and immunoblotting using anti-ST^A, ST^C and mAb (data not shown).

Flow cytometry and immunoblotting

About 5×10^5 cells were incubated for 45 min at 0° with 5–20 μg of each mAb or 5 μl of E4.3 ascites together with 100 μl of

Dulbecco's phosphate-buffered saline (DPBS) containing 0·5% bovine serum albumin (BSA). Ten micrograms of mouse non-immune IgG (Cappel) was used as a control. The cells were washed in DPBS containing 0·5% BSA and treated with 5 μg of FITC-labelled second antibody in 100 μl of DPBS containing 0·5% BSA. After 30 min, the cells were washed twice with DPBS containing 0·5% BSA, and fixed with PBS containing 0·5% paraformaldehyde. The samples were analysed on an Epics Profile II (Coulter, Hialeah, FL) within 3 days.

Immunoblotting was performed by the method of Towbin.[37] Conditions for the staining of MCP were as described elsewhere.[35]

Factor I–cofactor assay

Methylamine-treated DACM-labelled C3 [f-C3(MA); 10 μg] was used as a substrate for factor I (0·5 μg).[38] The fluorescence is a marker for the C3d portion of C3.[38] The membrane form of MCP expressed on CHO cells was purified as described previously.[35] The soluble forms were prepared as follows. The supernatants of CHO cells expressing the soluble forms were pooled and the soluble MCP was immunoaffinity purified using M177-coupled Sepharose. The eluate containing MCP was dialysed against PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM EDTA–2Na, and 25 mM iodoacetamide, pH 7·5. No solubilizer was used throughout the purification, which is different from the reported procedures for membrane MCP purification.[35] The MCP concentration was determined by sandwich ELISA as reported previously.[16] A mixture of f-C3(MA), factor I and MCP (2–20 ng) was incubated for 3 hr at 37° in PBS containing 0·02% Nonidet P-40 (NP-40), then resolved by SDS–PAGE (8% gels) under reducing conditions. Cofactor activity was evaluated from the fluorescence intensity of the α chain and α_1 fragment using a spectrofluorometer (Hitachi F2000; Hitachi Co., Tokyo, Japan).[35,38]

MV H protein-mediated GME aggregation

A haemagglutination assay was performed as described elsewhere.[39] GME (25 μl of a 1% solution) were incubated with mAb (2 μg/ml) against MV H protein, MCP (E4.3, M177 and M160) or soluble MCP (30 μg/ml) for 30 min at 37° in U-bottomed 96-well plates. Serially diluted H protein (25 μl; Toyoshima strain; Osaka University, Osaka, Japan)[40] was then added and futher incubated for 45 min at 37°. After a short centrifugation (500 *g*, 5 min), the GME aggregation titre was judged under a microscope, and the effect of the mAb or soluble MCP on the MV H protein-mediated aggregation was determined.[39]

Assay for determination of MV infectivity

Monolayers of CHO^+ or Vero cells in 24-well plates were incubated at 37° for 60 min with soluble MCP (50 μg/ml) or anti-MCP mAb (10 μg/ml) in Ham's F12/10% FCS or DMEM/5% FCS, then infected with MV at a multiplicity of infection (MOI) of $1 \times 10^4 \sim\sim 0{\cdot}1$ plaque-forming units (PFU) (as determined on Vero cell monolayers) per well for 2 hr at 37°. The cells were washed three times and cultured for 3 days.[41] In some experiments, the basic medium contained 0·5% agarose (Takara H14; Takara Co., Kyoto, Japan). At 2–4 day intervals, the cytopathic effect was photographed using a Nikon inverted microscope. Production of MV H protein was confirmed by a rosette assay using GME, as described elsewhere.[39] This was further confirmed by flow cytometry using an anti-H mAb (data not shown).[21]

RESULTS

Production of recombinant soluble forms of MCP

Two recombinant forms of soluble MCP were produced from CHO cell transfectants, composed of four SCR, ST^C and UK (deleted TM and CYT) (γMCP1), and four SCR only (γMCP2). These forms are illustrated in Fig. 1. The γMCP1 gave a single high-performance liquid chromatography (HPLC) peak and a broad 45 000 MW band similar to the 47 000 MW soluble form on SDS-PAGE.[19]

Although the message and intracellular protein product of the γMCP2 was present, it was barely detectable in the supernatant of the CHO cell transfectant, suggesting the importance of the ST and UK regions for the stable expression of MCP (data not shown). We thus decided to use γMCP1 as the recombinant soluble form throughout the study.

C regulatory function of the recombinant soluble forms of MCP

MCP serves as a cofactor for factor I and thereby facilitates the proteolytic inactivation of C3b to C3bi.[1,2] The specific activity of the recombinant soluble form, γMCP1, was determined compared with a membrane form purified from the CHO^+ cells (Fig. 2). The two forms had similar factor I–cofactor activity. M75 or M177, whose epitope is mapped on SCR2, blocked cofactor activities of both forms of MCP almost completely (Fig. 2). MH61 or M160, which recognize SCR3, partially inhibited MCP cofactor activity. The mAb recognizing SCR1

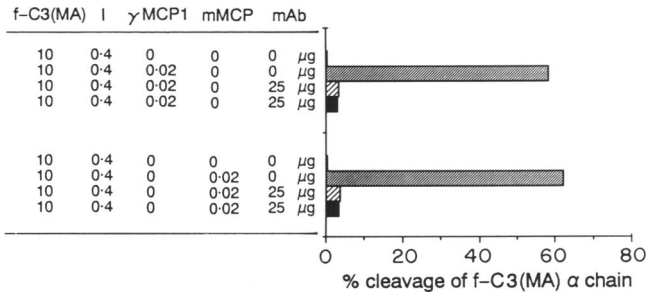

Figure 2. Determination of the cofactor activity of soluble and membrane MCP expressed by CHO cells. Factor I and f-C3(MA) were incubated with 20 ng of various forms of MCP as indicated, with or without mAb M75 (hatched bars) or M177 (closed bars). The buffer contained 0·02% NP-40. Cofactor activity was evaluated by measuring α–α_1 conversion using a fluorescent spectrophotometer as described elsewhere.[31] Although not shown in the figure, an inhibitory effect by the mAb on MCP cofactor activity was observed with various amounts (2–20 ng) of MCP of CHO cell origin and, under the conditions indicated (20 ng MCP, 25 μg mAb), M160 and MH61 allowed 35·0–39·4% cleavage. J4–48, S19S and E4.3 had no inhibitory effect. Three experiments were performed and a representative is shown. mMCP, solubilized membrane MCP (ST^C/CYT2 phenotype) from CHO cell transfectant.

Table 2. Mean fluorescence shifts by anti-ST^A and -ST^C

Antibody	Vero	GME	CHO^+
Rabbit IgG	0·861	0·620	0·846
Anti-ST^A	1·354	0·921	0·810
Anti-ST^C	3·050	2·286	2·683

of human MCP (Table 1) did not inhibit the cofactor activity (data not shown). Based upon the repetitive kinetic and blocking assays, the properties of factor I-mediated C3ma cleavage were similar in the soluble and membrane forms (Fig. 2).

The spectra of the reactivities of monkey cells with antibodies against human MCP

The reactivity of mAb against MCP with Vero and CHO^+ cells is shown in Table 1. All mAb tested reacted not with intact CHO or CHO^- cells (data not shown) but with CHO^+ cells expressing the ST^C/CYT2 phenotype of MCP. The four mAb mapped on the epitopes SCR2 or SCR3 recognized the protein on Vero cells, presumably the monkey MCP homologue. None of the mAb recognizing SCR1 reacted with any membrane protein. Based upon the reactivity of anti-ST^A and anti-ST^C monospecific antibodies, the Vero cell MCP possessed ST domains similar to those of humans (Table 2). In contrast to human erythrocytes, which have no MCP,[42] GME expressed a sizeable amount of the anti-human MCP-recognizable protein, the reaction spectrum of which was similar to that of Vero cells.

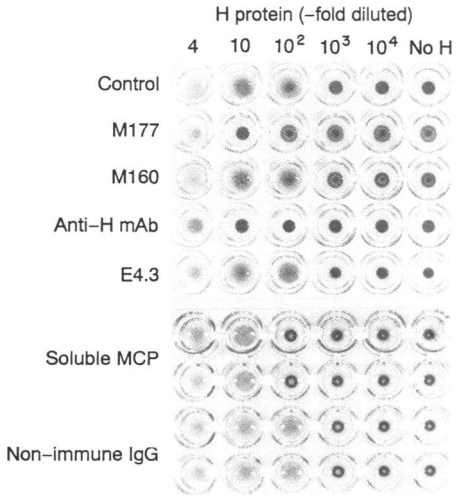

Figure 3. Effect of anti-MCP mAb and soluble MCP on the MV H protein-mediated aggregation of GME. GME was incubated with constant amounts of the indicated mAb (2 μg/ml) or γMCP1 (30 μg/ml), then serially diluted MV H protein was added. Non-immune mouse IgG (2 μg/ml) was used as a control (top lane and bottom two lanes). At intervals, the wells were observed under a microscope to assess cell aggregation. As shown in the figure, a 10^3-fold diluted MV H protein could not induce cell aggregation in the control lanes. Although not shown, variable amounts of mAb were used for this study, and 0·2 μg/ml of M177 or M75 was sufficient for one dilution unit of inhibition. Two experiments using different lots of GME were performed and the same results were obtained.

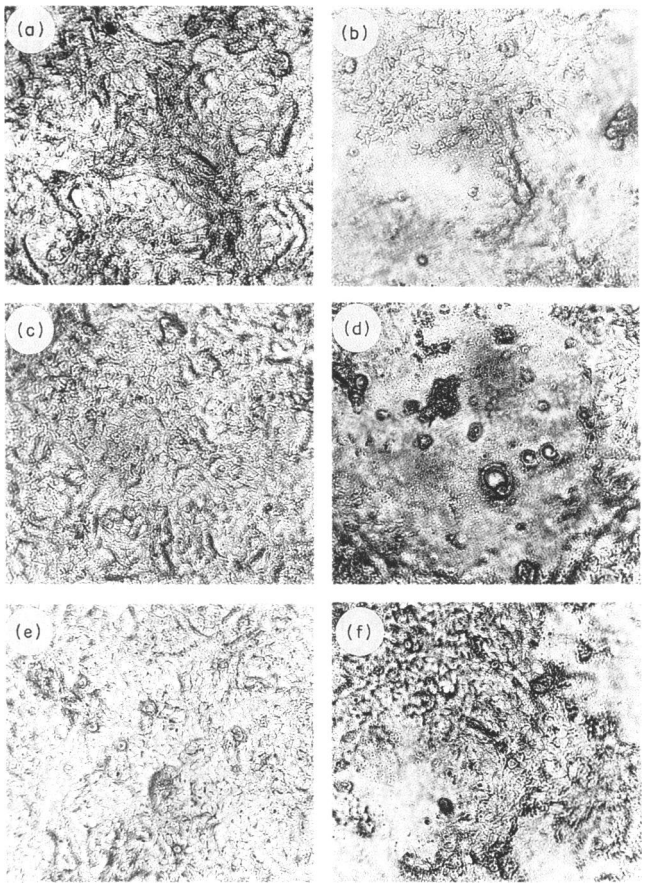

Figure 4. Cytopathic effect of MV induced on Vero cells in the presence of mAb or γMCP1. (a) Vero cells incubated with 10 μg of 243R (a mAb against CR1, cross-reactive with monkey CR1). (b) Vero cells incubated with 243R and MV. (c) Vero cells incubated with M177 (10 μg) and MV. (d) Vero cells incubated with M160 (10 μg) and MV. (e) Vero cells incubated with γMCP1 (50 μg). (f) Vero cells incubated with γMCP1 and MV. M160 is a mAb that only partially blocks the cofactor activity of MCP, while M177 blocks it completely at the dose used here.[24]

Inhibitory activity of soluble MCP on MV infection

The binding of soluble MCP to MV was measured in two ways. First, partially purified multimeric MV H protein (Toyoshima strain),[40,43] which was serially diluted, was incubated with GME in a buffer containing a constant amount of soluble MCP. The results were compared with those obtained with GME plus mAb against MV H protein, and mAb against MCP, E4.3, M160 and M177. The inhibitory degrees of GME rosette formation are shown in Fig. 3, suggesting that 30 μg/ml of γMCP1 blocked one dilution unit (10-fold) of MV binding, which corresponded to an inhibitory titre of 0·2 μg/ml of M177 or 0·3 μg/ml of anti-H mAb (data not shown). No inhibitory effect was observed using this assay when less than 30 μg/ml of γMCP1 was used.

The infectivity of MV was barely affected in the Vero

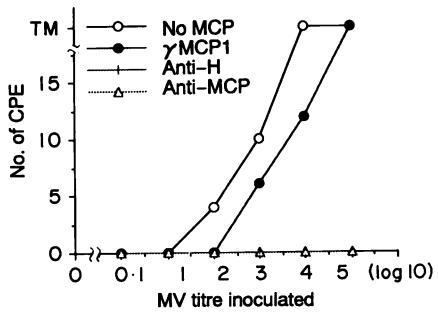

Figure 5. Effect of the recombinant soluble MCP on MV infectivity in CHO cells expressing MCP. Monolayer CHO^+ cells were inoculated with the indicated amounts of MV for 4 days at 37° in the presence or absence of γMCP1 (50 μg/ml), anti-H mAb (10 μg/ml) or M75 (10 μg/ml). The numbers of syncytia formed were calculated. TM in the *y*-axis means 'too many to calculate'. Four experiments were performed and a representative is shown. CPE, cytopathic effect.

system by the soluble MCP (50 μg/ml), which was simultaneously administered with MV (Fig. 4). Under our conditions, M177 (Fig. 4) and M75 (data not shown) blocked MV infection completely, whereas M160 and E4.3 did not. Anti-MV H protein also blocked MV infection (data not shown). On the other hand, MV-mediated syncytium formation induced upon CHO cells expressing human MCP was slightly blocked by 50 μg/ml γMCP1 (Fig. 5). M177 and M75, as well as anti-MV H, prevented CHO^+ cells from MV infection, consistent with the results using Vero cells.

Similar results were obtained with the MV that had been incubated with soluble MCP prior to infection (data not shown).

DISCUSSION

This is the first study to demonstrate that a recombinant soluble form of MCP, which blocks the C regulatory activity of MCP, can block MV infection. MV induces encephalitis with a relatively high frequency, namely post-measles encephalitis, inclusion body encephalitis, and subacute sclerosing panencephalitis (SSPE).[44] The fact that soluble MCP is present as monomers[19] and is relatively small is convenient for general therapeutic use. Furthermore, it may be possible to increase the potency of soluble MCP for targeting MV, either by creating multivalent active sites or by modulating the binding affinity by site-directed mutagenesis.

M177 and M75 blocked MV infection as effectively as anti-MV H protein antibody. Both M177 and M75 can block the factor I–cofactor activity of MCP.[19] Thus, the possibilities are that the essential site for MV binding is structurally in close proximity to that for C3b–C4b binding, that the binding of these mAb to MCP hinders these binding sites, that the mAb bind more than two functional sites in the primary sequence of MCP, and that MV binds target cells through the C3b–C4b deposited on the target cells, i.e. C3b or C4b deposited on MV bridges the MV and the MCP on target cells. Regarding the last possibility, most viruses including MV can activate the host C system, and C3b or C4b is deposited on the virus.[45,46] However, we consider that MV is not involved with C3b–C4b-mediated infection, since MV infection is induced in the absence of C (data not shown). The third possibility is unlikely, because no homologous sequences of more than four amino acids are present in the two separate portions in the MCP primary structure.[9] We currently hold that MV directly binds MCP-bearing target cells, and that the MV binding site is in close proximity to or partly overlaps the C3b–C4b binding sites in the primary structure of MCP (Fig. 1). No domain other than SCR is essential for MV infection in MCP.[47] Determining the epitopes of these mAb would be important for localizing the functional sites of MCP and settling the issue discussed above, which is in progress in our laboratory.

GME, but not human E, form rosettes with MV-infected cells that express MV H protein.[39] This rosette assay has traditionally been used as a test of MV infection.[39,40,43] Since GME, but not human E, possess the molecule recognized by anti-MCP, as shown in this study, the rosette formation reflects the molecular interaction sustained by the GME, MCP analogue and the MV H protein expressed on the virus-infected cells.

The γMCP1 (1) weakly blocked H protein-mediated GME aggregation, but (2) barely inhibited the infection of Vero cells with MV, although it significantly suppressed infection with MV of CHO cells expressing human MCP. The difference in the MCP density on the membranes may not explain the different γMCP1-dependent susceptibility of these cells to MV, because the expression levels of MCP in the CHO^+ and Vero cells were not very different (Table 1). Based on our studies, MV infectivity will not be affected by MCP density if the cells have more than 10^4 copies/cell of MCP. A probable explanation for the first issue is that MV have multiple fusion units[48] that sustain the binding to MCP, and it is impossible for soluble MCP to block all of the units perfectly. Alternatively, another membrane constituent plays a part in MV infection in addition to MCP.[41,49] Regardless, these results resemble those of soluble CD4 in human immunodeficiency virus (HIV) infection.[50] The second issue may reflect a difference between human and monkey MCPs. The ligand MV H protein binds with higher affinity to the monkey membrane MCP than to human soluble MCP, and reinforces the notion that differences in the primary structures between human MCP and the monkey MCP homologue, which is probably dominant in SCR1 (Table 1), are related to the susceptibility of cells to MV. The cloning of the Vero cell MCP is in progress in our laboratory.

The soluble form of MCP is reportedly present in body fluids including seminal plasma,[51] blood plasma,[15,23] and tears.[16] The concentrations, however, are too low to block MV infection.[16,51] However, it is possible that the soluble forms act efficiently on MV at a local environment. Since we have no *in vivo* report regarding this issue, the role of these soluble forms remains to be clarified.

MV, as well as HIV, suppresses the host immune system via the infection of T cells.[52,53] Exploring a way to block MV infection may complement work on the suppression of HIV infection. From this viewpoint, it is important to elucidate the mechanism whereby MV suppresses the immune system and how this aberrance can be prohibited. This could be intriguing work in the future.

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