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Opsonization-enhanced phagocytosis of foot-and-mouth disease virus

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SUMMARY

Using isolated peritoneal adherent cells, in which monocytes and macrophages dominate, the uptake and destruction of foot-and-mouth disease virus (FMDV) was enhanced by the opsonization with mAb of particular epitope specificity. This was seen under conditions in which virus infectivity was not neutralized, as determined by *in vitro* assay. Activation of macrophages *in vivo* further enhanced the uptake of opsonized virus, presumably by increasing the percentage of phagocytosing cells. The enhanced phagocytosis required opsonization and apparently made use of FcR^+ cells, because pepsin-treated antibodies and separated $F(ab')_2$ fragments did not enhance the capacity of the peritoneal cells to react with the virus. The reaction also relied on active phagocytosis, because inhibition of phagocytosis using silica interfered with the binding of both virus alone and virus/antibody complexes. This evidence shows that the previous *in vivo* observations (McCullough *et al.*, 1986b) of enhanced protection by the mAb can be related to active phagocytosis of virus and virus/antibody complexes. The reaction is not passive adsorption to the monocyte surface, but an active phagocytosis of the virus or the complex.

INTRODUCTION

The protective immune response against foot-and-mouth disease virus (FMDV) has, in the past, been related to levels of neutralizing antibody activity. This relationship has not always proven reliable (Van Bekkum, 1969; Sutmoller & Vieira, 1980; Pay *et al.*, 1983.). Recently we have demonstrated, using monoclonal antibodies (mAb) against particular epitopes on the surface of FMDV, that opsonization with a minimum affinity in the absence of neutralization of virus infectivity is sufficient to protect animals *in vivo* (McCullough *et al.*, 1986b). If the animals are treated with silica, or if the F_c portion of the antibody is removed, this opsonization-dependent protection is not seen. It therefore seems plausible that the mechanism by which the antibody was rendering the virus inactive in its capacity to produce disease was through the enhancement of the phagocytosis reaction. In order to determine if this proposal was true we looked at the capacity of syngeneic (with the mAb) peritoneal monocytes to react with FMDV before and after opsonizing with the mAb. We demonstrated that there was indeed an enhancement of the phagocytosis and that this could be abrogated by removal of the F_c portion of the antibody or by pretreatment of the monocytes with silica. This confirms our proposal that the likely mechanism by which antibody-mediated protection against FMDV is effected is through the opsonization-enhancement of phagocytosis and is not necessarily dependent upon neutralization of virus activity.

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MATERIALS AND METHODS

Virus

The O_1 Switzerland 1965 isolate of FMDV (World Reference Lab., Pirbright) was propagated in BHK 21 cells and purified by sucrose density centrifugation using a modification of the method of Brown & Cartwright (1963) as described by McCullough & Butcher (1982). Virus was labelled with [^{35}S]methionine and [^{3}H]uridine (Amersham International PLC) as described by McCullough *et al.* (1987b).

Monoclonal antibodies

The mAb against the O_1-Switzerland 1965 isolate of FMDV were as described by Brocchi *et al.* (1983) and de Simone *et al.* (1983). The characterization of these antibodies has been described by McCullough *et al.* (1986a,b, 1987a,b). The mAb were purified from ascites fluid prepared in BALB/c mice using a BioRad MAPS (Bio-Rad Laboratories Ltd) column as described by McCullough *et al.* (1986b).

Monocyte preparations

BALB/c mice peritoneal exudate cells were used as sources of monocytes. The peritoneal exudate cells were used either unstimulated, or 3 days after stimulation *in vivo* with 5 μg/mouse *E. coli* lipopolysaccharide (Sigma) or 1 ml/mouse of aged thioglycollate broth (Difco). The cells were removed from the peritoneal cavity by washing the cavity five times with cold, serum-free RPMI medium and the cells held on ice until ready for use. The cells were then centrifuged at 250 *g* for 10 min at 4°. The cells were washed twice with cold serum-free medium and resuspended in prewarmed (37°) RPMI medium (GIBCO-

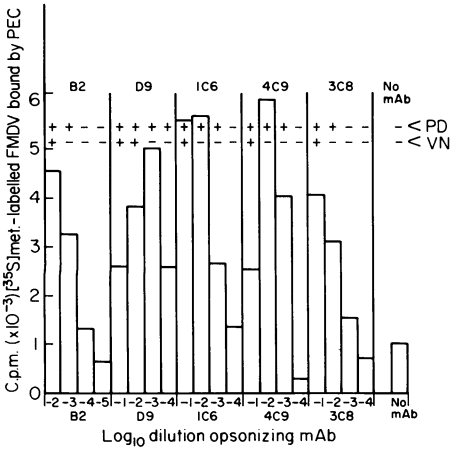

Figure 1. Uptake by non-stimulated BALB/c mouse peritoneal exudate cells of [^{35}S]methionine-labelled FMDV complexed with dilutions of the mAb B2, D9, 1C6, 4C9 and 3C8, and related to *in vivo* protection (PD) and *in vitro* neutralization of virus infectivity (VN).

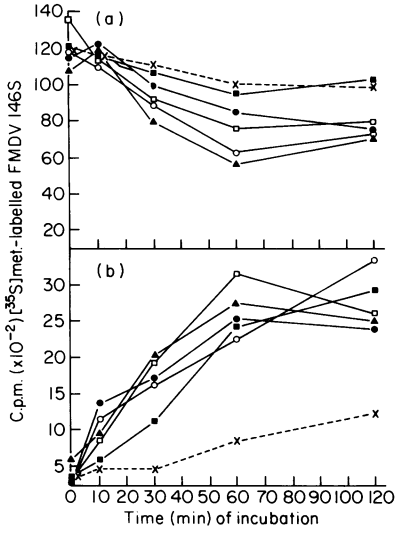

Figure 2. The kinetics of association of [^{35}S]methionine-labelled FMDV with non-stimulated BALB/c mouse PEC, as measured by the loss of radioactivity from the extracellular environment (a) and the increase of radioactivity associated with the PEC (b). (x), virus alone; (●), FMDV/B2 mAb complexes; (○), FMDV/D9; (■), FMDV/1C6; (□), FMDV/4C9; (▲), FMDV/3C8.

Europe Ltd) supplemented with 10% (v/v) fetal calf serum (FCS) and 2 mM glutamine. Although 1% (v/v) non-essential aminoacids and 1 mM sodium pyruvate were also added to the medium in the initial experiments, repetition of the work in the absence of these supplements demonstrated that they were unnecessary for the phagocytic assay.

Phagocytosis assay

Aliquots of 100 μl of the peritoneal exudate cells at a concentration of 10^7 cells/ml were mixed with 100 μl of purified FMDV at 1 μg/ml. This virus was labelled with either [^{35}S]methionine or [^{3}H]uridine. These mixtures were prepared in 1 ml Eppendorf

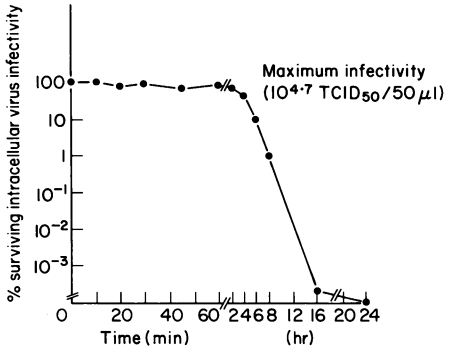

Figure 3. Kinetics of the loss of FMDV infectivity associated with non-stimulated BALB/c mouse PEC after incubation at 37° for 1 hr.

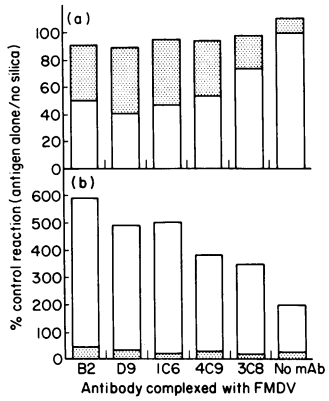

Figure 4. Percentage inhibition by silica of the phagocytosis of FMDV or FMDV/mAb complexes, measured as a percentage of the reaction with FMDV alone and no silica treatment. (a), extracellular counts; (b), cell-associated counts. Unshaded areas, phagocytosis in the absence of silica treatment; stippled areas, phagocytosis after silica treatment of the monocytes.

tubes pretreated with RPMI medium supplemented with 10% FCS. This pretreatment prevented the attachment of the monocytes to the plastic of the Eppendorf tubes. The mixtures were incubated in a waterbath at 37° for 1–2 hr unless otherwise indicated in the results section. At the end of the incubation the tubes were centrifuged on an Eppendorf centrifuge at maximum speed for 10 seconds. The supernatants were removed and the radioactivity counted on Whatman filter discs as described by McCullough *et al.* (1987b). The cell pellets were washed twice using the same method of centrifugation and then lysed by the addition of 100 μl of deionized water. The radioactivity associated with these lysed cell pellets was then estimated as for the supernatants.

Removal of the Fc portion of the mAb

$F(ab')_2$ fragments of each mAb were generated by pepsin digestion of whole molecules, using the procedures described by Turner & Bennich (1968).

Inhibition of phagocytosis by silica

Before addition of the virus, the freshly isolated peritoneal exudate cells were incubated with 1 mg/ml of silica (silicon

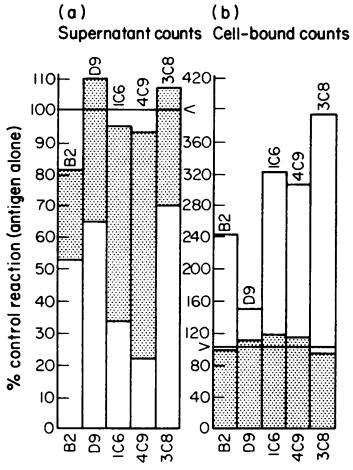

Figure 5. Influence of pepsin digestion on the capacity of mAb to enhance phagocytosis of FMDV, measured with respect to reduction in extracellular radioactivity (a), and increase in cell-associated radioactivity (b). Unshaded areas, phagocytosis in the presence of untreated mAb; stippled areas, phagocytosis in the presence of pepsin-treated mAb. 100% is the value obtained with FMDV alone (no mAb).

dioxide; Sigma, Poole, Dorset) for 2 hr at 37°. Control cultures were incubated for 2 hr in medium alone. The cells were then washed three times with serum-free RPMI medium by centrifugation at 250 *g*. These cultures were then resuspended in complete RPMI medium as for the normal peritoneal exudate cell cultures.

RESULTS

The influence of opsonization on the phagocytosis of FMDV

Figure 1 shows the relative uptake of radio-labelled FMDV, alone or after incubation with the different mAb for 1 hr at 37°, by non-stimulated peritoneal macrophages. Similar results were obtained with stimulated peritoneal cells, except that the counts with both free virus and opsonized virus were higher. There was an observable uptake of radio-labelled virus from the medium by the peritoneal exudate cells (PEC)-column marked 'no mAb'. This association of radioactivity with the PEC was enhanced by pre-incubation of the virus with mAb, and in this context several dilutions of the mAb were found to be effective. Figure 1 also shows that there was a consistent relationship between protection *in vivo* of neonatal mice and enhancement of phagocytosis *in vitro* by the mAb, but no such relationship existed with *in vitro* neutralization of virus infectivity. Indeed, it was enhanced phagocytosis by mAb, and not neutralization of virus infectivity, which appeared to be related to protection *in vivo*.

High concentrations of D9 (10^{-1} and 10^{-2} dilutions) and 4C9 (10^{-1} dilution) antibodies enhanced phagocytosis of the virus to a lesser degree than intermediate concentrations (10^{-3} dilution for D9; 10^{-2} dilution for 4C9). The number of molecules of D9 which bind per virion increases from 10^{-3} through 10^{-1} dilutions, resulting in a reduction in the size of the virus/antibody aggregates seen at the 10^{-3} dilution (J. R. Crowther and K. C. McCullough, unpublished data). Thus, for each antibody molecule associated with the phagocytes, the *relative* quantity of radioactive virus concomitantly associated would be less with the 10^{-1} and 10^{-2} dilutions. With the 4C9 mAb, virus structure is in the process of being destroyed during the time of incubation shown in Fig. 1 (McCullough *et al.*, 1987b). Thus, there would be less radioactively labelled protein associated with the 10^{-1} dilution of 4C9 than with the 10^{-2} dilution at the time of phagocytosis. Neither of these phenomena are seen with the concentrations of the other mAb, as shown in Fig. 1.

Kinetics of phagocytosis of FMDV before and after opsonization with mAb

Figure 2 demonstrates that with each mAb, it was not only the uptake of virus by the phagocytes which was enhanced by opsonization, but also the rate at which the cells bound the virus. Although this was not seen with all of the mAb when the radioactivity remaining in the supernatant medium was assayed, the cell-associated radioactivity clearly showed the increased kinetics after opsonization. In this context, the majority of the mAb were similar, with 50% of maximum uptake of virus being seen within 10–20 min, and maximum uptake within 60 min. This compared to at least 50 min and at least 120 min, respectively, for non-opsonized virus.

This association of the virus with PEC was not a passive reaction. The procedure appeared to be an active phagocytosis resulting in destruction of the virus. Kinetics of virus infectivity associated with PEC declined with time (Fig. 3). If live FMDV was incubated with PEC for 1hr at 37°, and the infectivity of cell-associated virus measured thereafter, this infectivity remained maximal for between 1 and 2 hr, and then declined rapidly. By 4 hr only 50% of the maximal infectivity remained, falling to 1% by 8 hr. Only 0·0002% was found by 16 hr, and nothing was detectable after 24 hr incubation. If the virus was incubated in the absence of phagocytes, but otherwise under the same conditions as shown in Fig. 3, there was a 10% reduction of virus infectivity during the first 8 hr, and a reduction to 40% after 24 hr (data not presented). Therefore, the decrease in virus infectivity seen in Fig. 3 cannot be explained by heat-lability of the virus.

Influence of silica treatment on phagocytosis by peritoneal cells

When peritoneal cells (stimulated or unstimulated) were treated with silicon dioxide in order to prevent phagocytosis, as described in the Materials and Methods, their capacity to phagocytose non-opsonized FMDV was impaired. Figure 4(a) shows that the phagocytosis of both non-opsonized and mAb-opsonized FMDV, as measured by the reduction of radioactivity in the medium (unshaded areas), was prevented by silica treatment of the monocytes (shaded areas). When the quantity of cell-associated reactivity was analysed (Fig. 4b), the enhanced uptake of radioactivity obtained through mAb-opsonization (unshaded areas) was not seen after silica treatment of the monocytes (shaded areas). The uptake of non-opsonized virus was also reduced by the silica treatment (to 10% of that obtained with untreated cells).

The requirement for the Fc portion of the mAb during opsonization

Using pepsin-treated mAb, it was shown that the mAb-enhanced phagocytosis of FMDV was dependent upon the Fc

portion of antibody (Fig. 5). Regardless of the mAb, pepsin pretreatment reduced their capacity to enhance the removal of radioactively labelled FMDV from the medium by peritoneal exudate cells. The amount of radioactivity remaining in the medium when pepsin-treated mAb were used (stippled areas) was closer to the control reaction of phagocytosis of virus alone (no antibody opsonization) than the amount remaining when untreated mAb were used (unshaded areas). Similarly, the amount of radioactivity associated with the cells approached the levels obtained with virus alone when $F(ab')_2$ preparations of the mAb were used. That is, the augmented uptake of radiolabelled antigen after opsonization with complete antibody molecules (unshaded areas) was not seen if the opsonization used pepsin-treated mAb (stippled areas—the values were no different from those obtained using virus alone).

DISCUSSION

Foot-and-mouth disease virus is a picornavirus, and typical of that group in that it does not induce the incorporation of virion proteins into the plasma membrane of infected cells. Furthermore, the virus produces disease symptoms within 3–5 days of infection. Hence, studies on protective immune responses have concentrated on humoral antibody activity. Although a relationship has been identified between circulating neutralizing antibody and protection (Mackowiak *et al.*, 1962; Van Bekkum, 1969; Sutmoller & Vieira, 1980; Pay *et al.*, 1983), this has not always proven reliable (Van Bekkum, 1969; Sutmoller & Vieira, 1980, Pay *et al.*, 1983). Recently, using mAb against different epitopes on FMDV, we have shown that it is not simply neutralizing antibody but opsonizing antibody which is important in the protective immune response against FMDV (McCullough *et al.*, 1986b). This work also demonstrated that the opsonization enhancement of immune protection against FMDV relied on active phagocytosis (silica-treated animals were not protected) and intact immunoglobulin molecules [$F(ab')_2$ fragments of the mAb were ineffective]. Consequently, it would appear that phagocytosis of opsonized FMDV by FcR^+ cells is a major effector immune defence against FMDV.

The present study was undertaken to elaborate upon this proposal. Cells known to be phagocytic, and likely to be FcR^+—peritoneal monocytes and macrophages—were shown to be capable of engulfing radiolabelled FMDV. When the virus was opsonized with mAb, the capacity of mononuclear cells to react with the virus was enhanced. The majority of this mononuclear cell-associated virus was found within the cytoplasm (after incubation of the phagocytes with virus, the cells were washed to remove extracellular virus, disrupted by freeze-thawing and centrifuged at 3000 *g* for 20 min; the majority of the radioactivity remained in the supernatant—data not presented). It would appear, therefore, that these mononuclear cells were phagocytosing the virus, and that the phagocytosis was enhanced by opsonization. This required both active phagocytosis and complete antibody molecules binding to Fc receptors, since silica-treated monocytes and $F(ab')_2$ fragments of the mAb were much less effective.

A number of observations, when taken together, demonstrated that the virus was not infecting the mononuclear cells. There was an enhancement of cell-association using mAb; *in vivo*-activated monocytes/macrophages were more efficient than resident peritoneal cells at interacting with virus; the kinetics of the disappearance of virus infectivity after mononuclear cell-association was different from that seen during infection of target host cells (BHK_{21} cell monolayers) (data not presented); and replication of virus (an increase in virus infectivity between 4 hr and 24 hr of the association with the cells—as seen after infection of BHK_{21} cells) was not found after mononuclear cell association (K. C. McCullough and D. Parkinson, unpublished data).

The *in vitro* phagocytosis could be related to the *in vivo* protection previously reported (McCullough *et al.*, 1986b) in that only at those dilutions which protected *in vivo* could the mAb enhance the phagocytosis of the virus. Consequently, phagocytosis of FMDV would appear to be an important immunological defence, both when the immune system confronts the virus and under conditions of antibody opsonization. This confirms the conclusions drawn from the *in vivo* work (McCullough *et al.*, 1986b), but that is not to say that the sole role of antibody in the immune defence against FMDV is as an opsonin. Under the appropriate conditions antibody of certain specificity and affinity can destroy the structure of FMDV (McCullough *et al.*, 1987b). It is probable that the immune defences against FMDV take the form of an opsonization of the virus, leading to enhanced phagocytic destruction (both in terms of the rate of phagocytosis and quantity of virus phagocytosed), but with the additional possibility of direct destruction of virus structure by antibody of certain specificity. The direct neutralization of virus infectivity by antibody *in vivo* is only of relevance if those antibodies are enhancing phagocytosis. It is opsonization of virus which is the important mechanism, probably related to the affinity of the antibody–epitope reaction (reviewed by Leslie, 1985).

These observations on the effector immune defences against foot-and-mouth disease should be considered when attempting to determine the immune status of animals, especially with respect to vaccination efficacy. In a move towards this, Hamblin *et al.* (1986a,b) have shown that the 'liquid-phase' ELISA, which will measure opsonising antibody (McCullough, Crowther & Butcher, 1985), will detect seroconversion in animals which is related to protection against subsequent virus challenge. This ability to detect truely protective antibody (opsonizing antibody, and not simply antibody which will neutralize virus infectivity *in vitro*) is most pertinent in the area of the 'peptide' vaccine, where the affinity and diversity (in terms of specificity) of the induced immune response will be limited.

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