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Antigenicity of Mouse Hepatitis Virus Strain 3 Subcomponents in C57 Strain Mice

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With 2 Figures

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Summary

C57 strain mice were inoculated intraperitoneally with denatured mouse hepatitis virus strain 3 particles and virus surface projection, membrane and ribonucleoprotein subcomponents, obtained from detergent treated purified virus preparations. All immunised animals developed high levels of serum antibody directed against the respective antigens, detectable by enzyme-linked immunosorbent assay. Mice that had been immunised with denatured virus particles or surface projections were protected against infection with mouse hepatitis virus strain 3, whereas immunisation with virus membrane or ribonucleoprotein subcomponents failed to protect mice against virus challenge.

Introduction

Mouse hepatitis virus (MHV) is a member of the Coronaviridae group of viruses which are all lipid-containing, enveloped, positive-stranded viruses that bud from endoplasmic reticular membranes (17, 23). The structural polypeptides of a number of MHV strains, including strains A59 (2, 21), JHM (1, 2, 25) and 3 (1, 12) have been described, and consist of 4 to 6 polypeptides of similar size and composition. These polypeptides are of 3 main types, with up to 3 high mol. wt. glycopolypeptides comprising the surface projections, up to 2 low mol. wt. polypeptides forming membrane proteins, and a single polypeptide of about 50,000 mol. wt. comprising the ribonucleoprotein (RNP) (12, 21, 25). Several reports have described the separation of some or all of the subviral components of MHV A59 (22) and other coronaviruses (4, 6, 14, 18) by disruption of virus particles with Nonidet P40 or Triton X100.

Most strains of mice can be infected with MHV 3 with the development of fulminant hepatitis, although numerous other organs are also infected (17). The

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susceptibility of the C57 mouse strain to MHV 3 has been studied previously in this (16) and other laboratories (26), with the first deaths occurring within 5 days after infection.

In this report we describe the isolation and purification of MHV 3 subviral components, and the immune response of susceptible C57 mice inoculated with inactivated MHV 3 particles and subviral components.

Materials and Methods

Virus Growth

MHV3 was grown in confluent secondary mouse embryonic fibroblasts. Monolayers were infected at an input multiplicity of 0.1 infectious particles per cell and following an adsorption period of 1.5 hours at 37° C, were incubated for 72 hours at 37° C in Eagle's MEM with 2 per cent foetal calf serum (13). Aliquots of this virus suspension were stored at −70° C and used for the preparation of purified virus particles and subcomponents.

Preparation of Purified Virus

Virus was purified at 0° to 4° C as described previously (13). The virus was pelleted at 75,000 × *g* for 1 hour and then resuspended in 1 ml Dulbecco's phosphate buffered saline "A" (PBSA). The resuspended virus was overlaid on to a linear 25 to 55 per cent (w/w) sucrose gradient in PBSA and centrifuged for 16 hours at 90,000 × *g*. The virus peak at 1.18 g/ml was collected.

Iodination Procedure

Preparations of purified virus particles were dialysed against PBSA for 16 hours at 4° C, and then disrupted with 1 per cent Nonidet P40 in PBSA at 21° C in order that all virus components were available for iodination. The iodination procedure used was based on that described by GREENWOOD *et al.* (7).

50 µl of 0.5 M sodium ^{125}I iodide in 0.5 M NaH_2PO_4 2 H_2O and 0.5 M Na_2HPO_4 buffer (phosphate buffer, pH 7.5) was added to 1 ml samples of dialysed, Nonidet P40 treated virus. 40 µl of chloramine T (1 mg/ml) in phosphate buffer was added for 30 to 60 seconds at 21° C. 40 µl sodium metabisulphite (1 mg/ml) in phosphate buffer was then added to stop the reaction.

Isolation of Purified Virus Subcomponents

Sucrose-gradient-purified virus particles were disrupted at 21° C with 1 per cent Nonidet P40 in PBSA and layered on to either 10 to 55 per cent (w/w) or 25 to 65 per cent (w/w) sucrose gradients in PBSA and centrifuged for 16 hours at 90,000 × *g* at 4° C. Subcomponent peaks were located at 1.13 g/ml in 10 to 55 per cent (w/w) sucrose gradients and at 1.23 and 1.27 g/ml in 25 to 65 per cent (w/w) sucrose gradients as described previously for HCV 229E (14).

Polyacrylamide Gel Electrophoresis

Iodinated virus and virus subcomponent fractions were treated with 5 per cent sodium dodecyl sulphate, 2 per cent 2-mercaptoethanol at 100° C for 1.5 minutes. A trace of bromophenyl blue was added to the reduced preparations, and the polypeptides were electrophoresed through 7.5 per cent polyacrylamide gels as described previously (15). After electrophoresis the gels were extruded and sliced into 1 mm discs and their radioactivity determined.

Immunisation Procedure

4—6 weeks old C57 BL/10 strain mice were obtained from the specific-pathogen-free (SPF) unit of this Centre. Groups of 10 mice were immunised with dilutions of purified denatured MHV3 particle preparations of titres about 10^7 ID_{50}, virus sub-

components derived from them, or PBSA. MHV 3 particles were denatured in 1:1000 formalin diluted in PBSA for 7 days at 4° C (11). The antigens were suspended in an equal volume of Freund's Complete Adjuvant and 0.1 ml volumes were injected intraperitoneally into the mice in two doses at 10 day intervals.

Challenge of Immunised Mice

At 20 days after immunisation, mice were challenged intraperitoneally with 0.1 ml of different titres of infectious MHV 3. Control mice were challenged with PBSA.

Enzyme-linked Immunosorbent Assay

Flat-bottomed wells in polystyrene microtitre plates (Dynatech) were coated with duplicate 0.2 ml amounts of antigen diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at room temperature. After incubation the plates were washed 4 times with phosphate-buffered saline containing 0.05 per cent Tween 20 and 0.02 per cent sodium azide (PBST) and shaken dry. Portions of 0.2 ml of sera diluted in PBST were added to the wells and incubated for 4 hours at room temperature. After 4 additional washes with PBST, 0.2 ml quantities of rabbit anti-mouse IgG antiserum (Miles Laboratories) at a dilution of $1:10^5$ was added and left for 3 hours at room temperature. The plates were then washed a further 4 times in PBST. After washing, goat anti-rabbit IgG antiserum labelled with alkaline phosphatase conjugate (Miles Laboratories) at a dilution of 1:800 was added in 0.2 ml quantities at room temperature overnight. After 4 additional washes with PBST, 0.2 ml of phosphatase substrate, consisting of a 0.1 per cent solution of p-nitrophenylphosphate in 10 per cent (w/v) diethanolamine buffer (pH 9.8) with 0.02 per cent sodium azide and 0.01 per cent $MgCl_2$ 6 H_2O, was added to each well. Absorbance values were read after 30 minutes at 405 nm in a Flow Titertek Multiscan photometer.

Results

Preparation of MHV 3 Antigens

Purified MHV 3 particles were obtained from sucrose density gradients as described previously (12). Subviral components were obtained by centrifuging Nonidet P40 disrupted MHV 3 particles on sucrose density gradients in a similar way to that described previously for the preparation of HCV 229E subcomponents (14). Surface projections were isolated on 10—55 per cent (w/w) sucrose density gradients at 1.13 g/ml, and membrane and RNP fractions were isolated on 25—65 per cent (w/w) sucrose gradients at 1.23 and 1.27 g/ml respectively. The purity of these subcomponents was determined by iodinating them and then analysing their polypeptides on polyacrylamide gels.

Fig. 1 shows a polyacrylamide gel of iodinated polypeptides from Nonidet P40 disrupted particles, which were similar to those obtained previously for unlabelled or ^{3}H-leucine labelled MHV 3 polypeptides (12). The polypeptides have mean mol. wt. of 170,000, 50,000, 22,000 and 20,000, and have been called VGP 170, VP 50, VGP 22 and VP 20 (VP, virus polypeptide; VGP, virus glycopolypeptide) (12). No polypeptide of mean mol. wt. 90,000, observed previously (12), was resolved, although it may be present but masked by the high background radioactivity.

Similar polypeptides have been obtained for other MHV strains (1, 19, 21, 25), and the polypeptides comprising the surface projections, membrane and RNP have been shown to correspond to VGP 170, VGP 22 and VP 20, and VP 50, respectively (12, 19, 21, 25).

Fig. 2a—c are polyacrylamide gels of MHV 3 subcomponent preparations isolated on sucrose density gradients from Nonidet P40 disrupted particles. The gels revealed a good separation of the structural polypeptides with the surface projection preparation containing VGP 170 (Fig. 2a), the membrane preparation VGP 22 and VP 20 (Fig. 2b), and the RNP preparation VP 50 (Fig. 2c). There was no evidence, from a number of polyacrylamide gels, of contamination of any of the subcomponents with other subcomponents, although there was considerable background radioactivity in all the gels of the iodinated subcomponents.

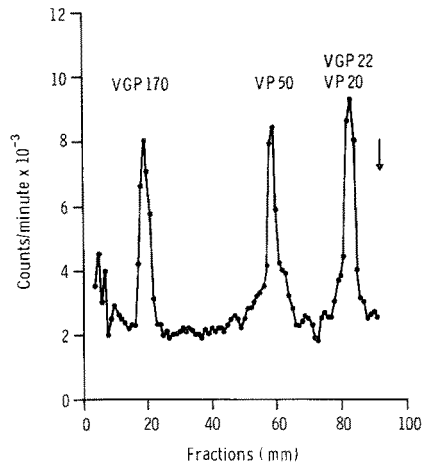

Fig. 1. Electrophoresis on a 7.5 per cent polyacrylamide gel of purified MHV3 particles disrupted with 1 per cent Nonidet P40 and labelled with ^{125}I before separation on a sucrose gradient. The arrow indicates the position migrated by the bromophenyl blue marker dye

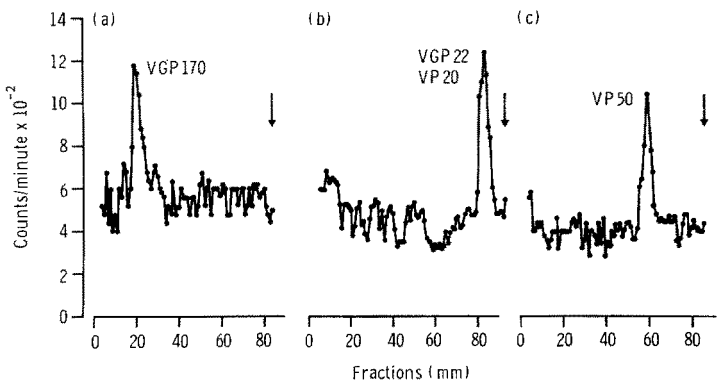

Fig. 2. Electrophoresis on 7.5 per cent polyacrylamide gels of purified MHV3 subcomponent fractions obtained from 1 per cent Nonidet P40 treated, ^{125}I labelled MHV3 particles. *a* Surface projections fraction, *b* membrane fraction, *c* RNP fraction. The arrows indicate the position migrated by the bromophenyl blue marker dye.

Antibody Responses in Mice Immunised with MHV 3 Particles and Subcomponents

Groups of 10 mice were each immunised with one of a number of dilutions of sucrose-gradient-purified inactivated virus particle or with subcomponent preparations. The corresponding dilutions of the MHV 3 subcomponent fractions contained comparable amounts of antigen as determined by ^{125}I labelling (Fig. 2). The mice were then observed for 20 days, and during this period no mice died or showed any symptoms of disease. Furthermore, sera were taken from them before inoculation, and 8, 14 and 20 days after inoculation, and antibody rises in the postinoculation sera were measured by ELISA using homologous antigens. Table 1 shows typical ELISA absorbance values obtained for sera taken 14 days after immunisation. For all these sera the ratios of postinoculation to preinoculation serum absorbance values at the same antigen and serum dilutions were significantly over 2, indicating that specific antibodies had developed (9, 10, 14). Thus, both virus particles and subcomponents elicited a significant antibody response. ELISAs using heterologous combinations of subcomponents and sera produced no antigenic reactions.

Table 1. *ELISA of purified MHV 3 antigens against mouse sera*[a]

MHV 3 antigens[b]	Antisera from mice given homologous antigen[c]	
	Preinoculation	Postinoculation
Virus particles	0.18	1.70
Surface projections	0.19	1.88
Membrane	0.23	0.61
RNP	0.11	0.38

[a] All readings were taken at 405 nm after 20 minutes
[b] Antigen dilution 1:50
[c] Sera from mice given 10^{-2} dilution of virus particles or virus subcomponents obtained 14 days after immunisation. Sera dilutions 1:200

Table 2 shows ELISA ratios, obtained for sera from animals immunised for 8, 14 and 20 days with a number of dilutions of denatured virus particles or virus subcomponents, tested against homologous antigens. Significant antibody rises, as measured by ELISA ratios, were observed in all sera from mice inoculated with denatured virus particles, although not in those from mice inoculated with subcomponent fractions. The antibody rises detected against purified subviral components were dose dependent and in all cases tested the membrane and RNP components were less immunogenic in mice than the surface projection components, even after repeated immunisation.

Challenge of Immunised Mice with Infectious MHV 3 Particles

Dilutions of 10^{-1} of denatured virus particles and subcomponents and an immunisation period of 20 days were selected from Table 2 as suitable for producing high levels of antibody in immunised mice. After immunisation, mice were

Table 2. *Antibody rises in sera from C57 strain mice immunised with denatured virus particles or virus subcomponents*

Days after immunisation	Immunising antigen dilution[a]	ELISA ratios using homologous antigens[b]			
		Denatured particles	Surface projections	Membrane	RNP
8	10^{-1}	7.5	1.0	0.8	0.9
	10^{-2}	7.7	1.0	0.9	1.5
	10^{-3}	11.1	1.3	1.2	1.3
14	10^{-1}	9.6	19.4	3.7	4.3
	10^{-2}	9.4	9.9	2.7	3.4
	10^{-3}	11.7	7.4	2.0	2.0
20	10^{-1}	9.8	17.1	5.1	4.5
	10^{-2}	6.0	8.9	3.2	2.9
	10^{-3}	6.6	6.4	2.1	1.8

[a] Immunising antigens were sucrose gradient purified denatured MHV 3 particles and MHV subcomponents. Each dilution contained comparable amounts of antigen

[b] ELISA ratios were the ratio of postinoculation to preinoculation serum absorbance values for antigen dilutions of 1:50 and serum dilutions of 1:200

Table 3. *Protection of immunised C57 mice against challenge with MHV 3*

Immunising antigen[a]	Titre of challenge MHV 3 (ID_{50}) per ml	Number of mice surviving after challenge (days)[b]			
		5	6	7	20
Formalin denatured particles	10^5	10	9	7	5
	10^4	10	9	8	7
	10^3	10	10	9	8
	PBSA	10	10	10	10
Surface projections	10^5	10	8	8	5
	10^4	10	8	8	7
	10^3	10	9	9	6
	PBSA	10	10	10	10
Membrane	10^5	4	0	0	0
	10^4	5	2	0	0
	10^3	3	1	0	0
	PBSA	10	10	10	10
RNP	10^5	2	2	0	0
	10^4	1	0	0	0
	10^3	2	1	0	0
	PBSA	10	10	10	10
PBSA	10^5	3	0	0	0
	10^4	4	1	0	0
	10^3	3	0	0	0
	PBSA	10	10	10	10

[a] Mice were immunised with purified inactivated MHV 3 particles diluted 1:10 in PBSA, purified MHV 3 subcomponents diluted 1:10 in PBSA, or PBSA; and then challenged with MHV 3 20 days later

[b] 10 mice were used for each dilution of challenge MHV 3

challenged with different titres of infectious MHV 3 or PBSA (Table 3). The mice were then observed daily for 20 days and the numbers surviving on each day noted. Between 5 and 8 out of 10 mice immunised with denatured virus particles, and between 5 and 7 out of 10 mice immunised with surface projections survived up to 20 days after challenge with MHV 3. All non-immunised mice were killed by 7 days after virus challenge by the MHV 3 dilutions tested, with the first deaths occurring by day 5. However, non-immunised mice challenged with PBSA showed no signs of illness. Mice immunised with purified membrane and RNP fractions were not protected against MHV 3 challenge — all of them died within 6 days.

Discussion

In this paper we report the isolation and purification of MHV 3 subviral components and have shown the role of each subcomponent in the protection of immunised mice against challenge with infectious MHV 3. It was difficult to ensure that mice immunised with different virus subcomponent preparations all produced comparable amounts of antibody, as there was considerable variation in the immunogenicity of the subcomponents. The highest antibody rises detected by ELISA were directed against surface projections, while lower antibody rises were observed against membrane and RNP, suggesting that the most immunogenic part of the virus is an antigen(s) associated with the surface projections. Similar results have been obtained previously with human coronaviruses (14) and the porcine coronavirus transmissible gastroenteritis virus (TGEV) (5).

We have shown a close correlation between the protection of mice against infection and antibody rises in inactivated virus particles and surface projections. Furthermore, experiments have been reported showing that rabbits and sows inoculated with whole TGEV particles or surface projections may be protected against virus challenge by the stimulation of neutralising antibody (5). Similar properties have been observed for the surface projections of other lipid-containing, enveloped RNA viruses (3, 8, 24). Our results with MHV 3 membrane and RNP subcomponents suggest that although antibody was induced by these subcomponents in mice, this antibody was produced in relatively low amounts and had no detectable protective effect against infection. Repeated immunisation of mice with membrane and RNP subcomponents did not lead to increased amounts of antibody or to any protection against MHV 3 infection. Thus, it is unlikely that these subcomponents were protective and this protection was missed due to the poor immunogenicity of these components.

There was no decline in the antigenicity of subcomponents after Nonidet P40 treatment, dialysis or centrifugation in sucrose gradients (Hasony, unpublished data) as has been shown for other viruses, including herpesviruses (20). In our ELISAs, only IgG antibodies were measured in the postinoculation mouse sera, although other immunoglobulin classes may have important roles in the protection of mice against infection. However, by the 20th day after immunisation, IgG antibodies should be the most common antibodies present and have the predominant role in protection against infection.

Further studies are in progress to extend these experiments to other coronaviruses and to determine the antigenic and structural relationships between coronavirus subcomponents.

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