Table 2 Effect of immunosuppression on autoantibody titres in reovirus-infected mice

Groups		Autoantibodies to								
		Cytoplasmic antigens*			Surface antigens†			Hormones‡		
Infection	ALS	Anterior pituitary	Islets	GH_3	Islets	GH_3	Thymocytes	Insulin	GH	Antibody to reovirus
+	−	32	8	16	4	2	8	56	89	256
+	+	2	2	<2	<2	<2	<2	16	20	16
−	+	<2	<2	<2	<2	<2	<2	0	0	<8
−	−	<2	<2	<2	<2	<2	<2	0	0	<8

Reo-1 infection and ALS treatment were as in Fig. 1. Autoantibodies were measured in pooled sera from 10 mice. Similar results were obtained with pooled sera from two other experiments. Titres for reovirus antibody are given as reciprocal antibody titres by haemagglutination-inhibition tests to reo-1 in pooled sera 17 days after infection.

* Bouin's-fixed sections of anterior pituitary and pancreas from uninfected mice, or acetone-fixed rat GH_3 cells were incubated with serial dilutions of mouse sera (obtained 14 days after infection) and stained with fluorescein-labelled anti-mouse IgG. Titres represent the reciprocal of the highest dilution of sera giving positive fluorescence[10].

† Suspensions of cultured mouse SJL pancreatic islet cells, or rat GH_3 cells, or freshly prepared thymocytes from weanling SJL mice were incubated with pooled mouse sera obtained 14 days after infection and stained with biotin-conjugated anti-mouse immunoglobulin followed by fluorescein-labelled avidin[16]. Titres represent the reciprocal of the highest dilution of sera giving positive fluorescence.

‡ Individual sera from 10–20 mice per group (obtained 17 days after infection) were tested for antibodies to insulin and GH by an enzyme-linked immunosorbent assay[10]. Sera with titres >10 were considered positive and numbers represent per cent positive.

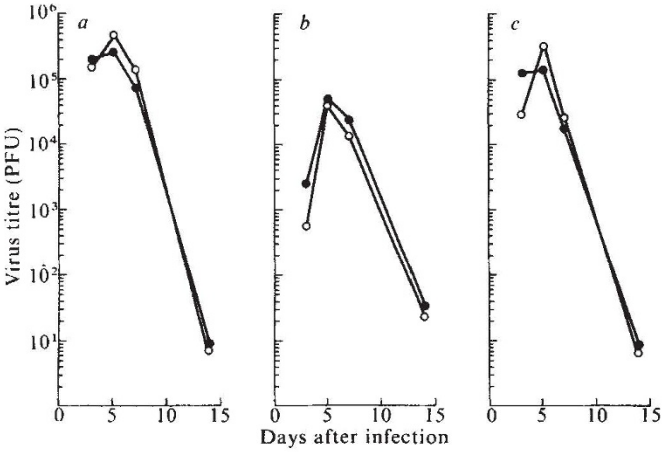

Fig. 2 Reovirus titre in ALS treated mice. SJL mice were infected with 5×10^5 PFU and treated with ALS as in Fig. 1. Virus titres in homogenates of liver (*a*), heart (*b*) and pancreas (*c*) were measured at different times after infection. Titrations were performed on L929 cells by a standard plaque assay[10] and expressed as PFU per organ. Each point represents the mean titre of two to four individual mice. ○, Infected mice given ALS; ●, infected mice not ALS.

virus alone nor sub-diabetogenic doses of streptozotocin alone could produce hyperglycaemia, whereas treatment with both agents resulted in overt diabetes[21]. Our interpretation was that neither virus nor streptozotocin alone depleted the β-cell reserve sufficiently to result in diabetes. Reovirus-induced polyendocrine disease may also be the result of cumulative injury. Both the direct lytic effect of the virus and autoimmune pathology may be required to decrease the hormone reserve sufficiently to make the disease overt. The possibility that virus-induced autoimmunity contributes to the pathogenesis of other diseases merits investigation.

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Attenuated reovirus type 3 strains generated by selection of haemagglutinin antigenic variants

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Infection of suckling mice with mammalian reovirus type 3 results in fatal encephalitis associated with marked destruction of neurones[1–4]. The viral tropism specific to neural cells is determined by the reovirus haemagglutinin[3,4]. This haemagglutinin (HA), the σ1 polypeptide, is encoded in the S1 dsRNA segment[5,6]. In addition to determining cell and tissue tropism, the viral haemagglutinin determines humoral and cellular immune specificity[7–10], binding to cellular microtubules[11] and inhibition of cellular DNA synthesis[12]. To elucidate the structural basis for the functions of the reovirus type 3 HA, we have recently isolated and characterized several anti-HA monoclonal antibodies, which we have used to show that there are distinct functional domains on the reovirus type 3 HA[13]. One group of monoclonal antibodies neutralizes viruses while a second inhibits haemagglutination. Thus there are distinct HA functional domains involving haemagglutination and neutralization. One of the neutralizing monoclonal antibodies was used to select reovirus antigenic variants that were no longer neutralized by the monoclonal antibodies. These 'variant' viruses were tested for their neurovirulence by determining their capacity to replicate in mouse brains, leading to fatal encephalitis. We report here that all variants are markedly less virulent then the parental type 3 virus from which they were selected. These findings demonstrate directly the critical role of the viral haemagglutinin in neurovirulence and show that variant viruses that are altered at a site recognized by neutralizing antibody cannot grow efficiently in neural tissue.

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Reovirus serotype 3 viruses with antigenically altered HA proteins were selected by incubating the neurovirulent virus stock (Dearing strain) with excess neutralizing monoclonal antibody (designated G5). Viral plaques that appeared on the plates were isolated and passaged a second time in the presence of G5 antibody to eliminate clones that were not resistant to the antibody. Resistant viruses (variants) were then grown in mouse L cells to give virus stocks. Three variant viruses (A, F and K) isolated in this manner were tested for their relative ability to resist neutralization by the G5 monoclonal antibody (Table 1). As reported previously, the parental reovirus type 3 (Dearing) strain was neutralized efficiently by G5 (ref. 13). All three variants showed an altered response to G5 antibody: the F and K variants were totally resistant to the antibody while the A variant was neutralized to a level intermediate between that of the Dearing parent and the F and K variants. For example, the A variant was neutralized at 1:20 and 1:100 dilutions of G5, but the plaque reduction at 1:500 was of borderline significance. The Dearing strain was neutralized at all the dilutions tested. Previous studies have shown that the G5 antibody neutralizes the Dearing strain at dilutions up to 1:12,500 (ref. 13). In contrast to the results with the G5 monoclonal antibody, the three variants were indistinguishable from the parental reovirus type 3 Dearing virus in neutralization tests using a type-specific hyperimmune antiserum (Table 1). These data indicate that all three variants encode an haemagglutinin having antigenic alterations that are readily detected with monoclonal antibodies but not with a standard hyperimmune antiserum.

To determine if the variants were altered in terms of virulence, we compared them with regard to their relative ability to cause fatal neurological disease in suckling mice. The type 3 Dearing parent is highly neurovirulent; as few as 10 plaque-forming units (PFU) inoculated intracerebrally (i.c.) in suckling mice kill 50% of mice by 14 days while higher doses kill 100% (ref. 3; Table 2). To compare the neurovirulence of type 3 Dearing with the variant viruses, we injected varying doses of virus into suckling mice and determined the survival pattern for each infected group of animals (Table 2). The F and K variants had LD_{50}s (lethal dose for 50% of the population) values of 3×10^7 and $>3\times10^7$ PFU, respectively, while the A variant had an intermediate LD_{50} of 1.8×10^5 PFU. These results indicate that all the variants are at least 10,000 times less neurovirulent than the Dearing strain from which they were derived. Interestingly, the A variant that was intermediate in antigenic reactivity, also exhibited an intermediate LD_{50} (1.8×10^5) whereas the F and K variants, which were not neutralized by G5, showed the greatest reduction in virulence. These data suggest that the loss of antigenic reactivity with the G5 antibody is directly related to the neurovirulence.

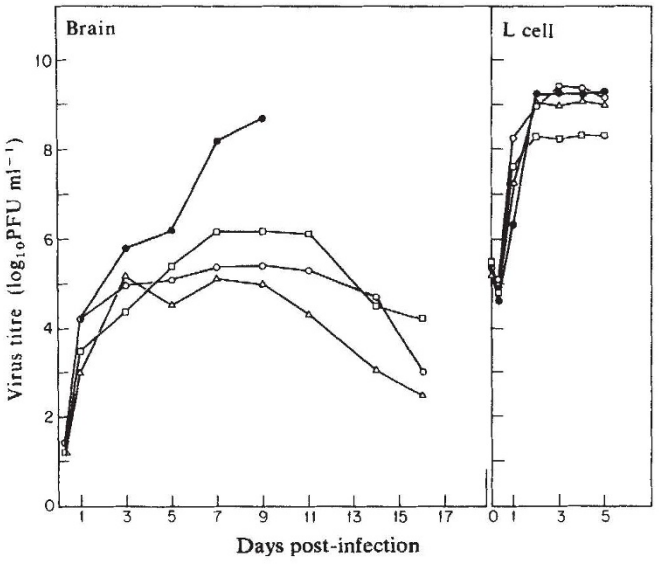

Fig. 1 Growth patterns of reovirus type 3 (Dearing, ●) and antigenic variants A (□), F(○) and K (△) in mouse brains (left) and mouse L cells in tissue culture (right). Virus growth in mouse brains was determined by injecting all mice i.c. with 1×10^3 PFU of virus. Three mice were killed at each time point and the virus titres in the individual brains were determined by titration of brain homogenates on mouse L cells at 37 °C as described previously[4]. Each value represents the average of three titrations. Virus growth in mouse L cells was determined by infecting monolayers of 2×10^5 cells at a multiplicity of infection of 5 PFU per cell. Triplicate samples were taken for titration at each indicated time point[16].

Table 1 Neutralization of reovirus serotype 3 (Dearing) and HA antigenic variants by monoclonal antibody and hyperimmune antiserum

Antibody	Dilution of antibody	Dearing strain	% Inoculum resistant to antibody Variant strains A	F	K
G5	1:20	0	21	100	88
	1:100	0	18	100	100
	1:500	5	48	100	100
Hyperimmune	1:200	6	7	4	3
	1:400	5	4	2	4
	1:800	14	4	5	9

Neutralization tests were performed as described previously[13] Briefly, dilutions of antibody were incubated with 100 PFU of virus at 34 °C for 1 h. These mixtures were then inoculated on to mouse L cells. The G5 antibody was used to select the variants and was used in these experiments as a purified IgG fraction[13]. Values represent the percentage of plaques observed in the presence of antibody relative to a phosphate-buffered saline control. The hyperimmune antiserum is a reference goose anti-reovirus type 3 antiserum obtained from the National Institute of Allergy and Infectious Diseases (V703-501-570).

Because neurovirulence is correlated with the ability of reovirus type 3 to replicate in suckling mouse brains, we analysed the growth of type 3 reovirus and the three variants in mouse brains (Fig. 1). The reovirus type 3 (Dearing) virus grows to high titres in the brain before death of the infected mice[3,4]. The virus yield in these experiments was $\sim1\times10^9$ PFU ml^{-1}, but titres were often $\geq1\times10^{10}$ PFU ml^{-1}. The three variants differed significantly from the Dearing strain in their ability to grow in mouse brains during the acute infection, particularly after the fifth day post-inoculation (Fig. 1). The F and K variants showed most reduction in growth while the A variant was again intermediate between the F and K variants and the Dearing virus.

To determine whether the inability of the three variants to grow in brains was due to a general defect of growth or was

Table 2 Neurovirulence of reovirus serotype 3 (Dearing) and HA antigenic variants A, F and K

Inoculum (PFU)	Dearing strain	% Mice alive on day 14 post-infection Variant strains A	F	K
1×10^1	50	100	100	100
1×10^2	0	100	100	100
1×10^3	0	100	100	100
1×10^4	NT	100	100	100
1×10^5	NT	90	100	100
3×10^5	NT	0	100	100
3×10^6	NT	0	90	100
3×10^7	NT	0	50	90
LD_{50}	10^1	1.8×10^5	3×10^7	$>3\times10^7$

Groups of CD mice (24–48 h old) were injected i.c. with varying doses of virus and observed for 14 days[3,4]. Animals began to die between days 8 and 10 in most cases. Values represent the percentage of mice alive at day 14 post-infection. NT, not tested. The LD_{50} is the dose of virus required to kill 50% of the animals in each group by day 14. LD_{50} values were calculated by the Reed and Muench method[15].

specific to growth in the brain, we examined the replication cycle of the variants and Dearing virus in mouse L cells (Fig. 1). Both reovirus type 3 Dearing and the three variants grew to a high titre. The variants were also not temperature-sensitive for growth in L cells (data not shown). Examination of brains infected with variants A, F and K showed a marked localization of tissue destruction in parts of the hippocampus. In contrast, infection with the Dearing strain caused tissue destruction not only in the hippocampus but also diffusely in the cortex and brain stem (unpublished data). Thus the pattern of restricted growth in the brain was not due simply to a general defect in the ability of the three variants to replicate, but to growth in restricted sites in the brain.

Thus, antigenic variants of reovirus type 3 selected by monoclonal antibodies directed against the major neutralization site, have markedly different levels of neurovirulence and capacity to grow *in vivo*. These experiments directly indicate that the reovirus type 3 haemagglutinin is a major determinant of neurovirulence, and imply that particular regions of the HA have critical roles in virulence. The site chosen for selection of the variants, the major neutralization determinant on the HA molecule, is probably the polypeptide region involved in binding to neurone receptors[14]. Other studies have shown that this region is also the site on HA that is recognized by cytotoxic T lymphocytes (unpublished data), and is involved in binding to cell receptors[14]. Furthermore, we have recently demonstrated that these variants induce cytolytic T lymphocytes as well as neutralizing antibody (unpublished data). Further data on the nature of the alterations in the haemagglutinin polypeptide of these variants should enable us to define the role of haemagglutinin in viral virulence more precisely. In addition, the use of monoclonal antibodies to generate attenuated viral strains may serve as a rapid and simple step for developing viral vaccines.

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Non-neutralizing monoclonal antibodies can prevent lethal alphavirus encephalitis

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Among the heterogeneous population of antibodies specifically induced during many acute viral infections, those having virus-neutralizing activity *in vitro* are generally considered to be most important for recovery and immunity to reinfection. Similarly, the ability to stimulate production of circulating neutralizing (NT) antibodies is a major criterion for evaluating the immunoprophylactic potential of many antiviral vaccines[1]. Although there is obviously an association between NT antibody induction and host resistance, we present here data which indicate that other virus-specific antibodies lacking NT function may be equally important in conferring protective immunity to alphaviruses. We used monoclonal antibodies against Sindbis virus (SV) to demonstrate that passive protection of SV-infected mice from fatal paralytic central nervous system (CNS) disease may be mediated not only by antibodies which neutralize the infectivity of extracellular virus particles but also by those lacking this capacity, which react preferentially with virus-infected cells.

Sindbis virus, the prototype of the alphaviruses, contains two distinct envelope glycoproteins, E1 and E2, which exist as a complex, and a nucleocapsid protein, NC. It has been shown that antibodies raised in rabbits against each of the individually isolated and purified glycoproteins of SV possess distinctly separable activities *in vitro*[2]. Antibodies to E1 (the viral haemagglutinin) inhibit haemagglutination by SV, whereas only antibodies to E2 significantly neutralize viral infectivity. Antibodies specific for the internal NC do not react with intact virions. Both haemagglutination-inhibiting (HI) and NT antibodies are induced during productive infections with SV as well as with other alphaviruses[3], but their respective roles in mediating host recovery are not clear.

Recently[4], we described the preparation of a large panel of monoclonal antibodies against the parental AR339 strain of SV (SV_p), some of which were used to define serological and biochemical relationships between SV and certain other alphaviruses. In the present study, six anti-E1, six anti-E2, and one anti-NC monoclonal antibodies were selected for further characterization and each was assessed for its protective capacity *in vivo*. Because, by any route of inoculation, SV_p produces an abortive immunizing infection in adult mice, a neuroadapted (na) variant[5] of SV_p was used to intracerebrally (i.c.) challenge C57BL/6J (B6) mice previously given monoclonal antibodies. When given i.c. to non-immune adult B6 mice, SV_{na} produces paralysis and fatal encephalitis.

To prepare monoclonal antibodies, spleen cells from BALB/cJ mice immunized with infectious SV_p were fused with P3X63-Ag8 myeloma cells, and hybrid cells were cloned by limiting dilution in hypoxanthine–aminopterin–thymidine medium[6]. Clones producing antibodies to SV_p were selected by solid-phase radioimmunoassay[7] of supernatants. Positive cultures were subcloned in soft agar and tested again by radioimmunoassay for anti-SV activity. Individual hybrid clones were expanded *in vitro*, and then inoculated into groups of pristane-primed BALB/cJ mice[8]. The resulting ascitic fluids derived from each hybrid clone were pooled, frozen in aliquots, and used in subsequent experiments. The specificity of each monoclonal antibody was determined by radioimmunoassay reactivity with individual SV_p structural proteins which had been separated by isoelectric focusing as previously described[2]. Specificity was confirmed by immunoprecipitation of radiolabelled virus, followed by polyacrylamide gel electrophoresis.

The isotypes of the monoclonal antibodies were determined by an indirect immunofluorescence procedure in which glass slides bearing acetone-fixed, SV_p-infected BHK-21 cells were exposed sequentially to predetermined concentrations of monoclonal antibody, rabbit antiserum (Litton Bionetics) specific for individual mouse immunoglobulin heavy-chain isotypes (IgM, IgG1, IgG2a, IgG2b or IgG3), and fluorescein-conjugated goat

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