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B. N. Fields and Karen Byers

Phil. Trans. R. Soc. Lond. B 1983 **303**, 209-218
doi: 10.1098/rstb.1983.0094

Phil Trans. R. Soc. Lond. B **303**, 209–218 (1983)
Printed in Great Britain

The genetic basis of viral virulence

By B. N. Fields and Karen Byers

Department of Microbiology and Molecular Genetics, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115, *U.S.A.*

The precise genetic and molecular determinants of viral virulence are poorly understood. Genetic studies with influenza and reovirus have indicated that virulence is multigenic. The high frequency of mutation of RNA viruses can complicate genetic analyses of virulence, resulting in phenotypes that are difficult to interpret. The ease with which the reoviruses reassort genome segments has made it possible to isolate reassortants from parental viruses causing different patterns of animal disease. It has thus been feasible to show that each of the three outer capsid proteins plays a major role in the pathogenesis of animal infection: the viral haemagglutinin determines the specificity of the immune response and cell and tissue tropism; the μ1c protein plays a central role in determining yield at portals of entry as well as in differentiated tissues; the σ3 protein inhibits host macromolecular synthesis. Thus virulence is clearly multigenic, with each of the viral components playing distinct roles.

Introduction

The capacity of a virus to produce lesions, symptoms and death (its 'virulence') has long been considered to be one of the most important aspects of viral infection (Burnet 1955). Although it is clear that it is often easy to distinguish strains of high or low virulence (by determining their relative capacities to produce illness or death), it is often difficult to define a precise genetic basis for the differences in virulence. This is mainly due to the complexities of the infectious process. An infectious agent must enter a host, multiply at a primary site, spread (if it is a systemic agent), target to distant tissues, interact with the immune system, damage target cells, and eventually be released to reinitiate the infectious process (Mims 1982). Unlike most laboratory experiments, when we study the pathogenesis of infectious disease we are in fact studying not one but many experimental variables. It is thus not surprising that virulence has been found to be multigenic (i.e. no single viral gene is the sole determinant of virulence) (Rott 1979; Fields 1982).

In this review, we shall focus on those models that have attempted to dissect further the precise genetic determinants of viral virulence, particularly by the use of animal systems. Although studies in tissue cultures are simple, readily controlled and highly manipulable, they are often too far removed from the situation *in vivo* to be readily interpretable. We have thus excluded from this selected review any studies on 'virulence' that were performed in cell cultures.

The genetic approach for studying viral virulence

The segmented nature of the genomes of the mammalian reoviruses and the influenza viruses have permitted detailed genetic studies to be performed (Scholtissek 1978; Palese 1977; Fields 1982). More recent studies have been extended to a third group of segmented viruses, the

bunyaviruses (Bishop & Shope 1979). The approach used for the study of these viruses has been to study the pathogenesis of prototype strains that differ in virulence. To determine the basis for the differences in virulence, 'reassortant' viruses are generated containing different combinations of genome segments from the two parents. The pathogenesis of the reassortant viruses is studied to see if the observed differences are properties of one or more of the viral genes.

Studies of viral pathogenesis in animals

Since the early experiments of Burnet and Kilbourne and colleagues, it has been hypothesized that virulence of influenza is multigenic. Burnet introduced the concept of multiple virulence genes to explain influenza viruses. Kilbourne and colleagues clearly demonstrated that neither the haemagglutinin (HA) nor the neuraminidase of the neurovirulent A/NWS (H_0N_1) strain was exclusively linked to neurovirulence (Burnet & Lind 1954; Mayer *et al.* 1973). Furthermore, they showed that neurovirulence could be divided into (1) the capacity to initiate intracerebral infection and (2) the capacity of infection once initiated to produce lethal infection. Rott and colleagues extended these studies to avian influenza viruses. They were able to demonstrate, by using natural isolates, a strict correlation between the structure of the HA and pathogenicity for the chicken (Bosch *et al.* 1979). These studies *in vitro* indicated that only those isolates that cleaved to an infectious form in a broad spectrum of host cells are pathogenic. In contrast to these results with natural isolates, genetic studies on influenza virus recombinants seemed to lead to evidence for a polygenic nature of virulence (Burnet & Lind 1954; Mayer *et al.* 1973; Rott *et al.* 1976). Further analysis indicated that when genes are introduced from an apathogenic strain into the highly pathogenic fowl plaque, an exchange of any RNA segment of the pathogenic strain is able to modify pathogenicity (Rott *et al.* 1976). Additionally, recombinants obtained from non-pathogenic parent viruses can be pathogenic (Scholtissek *et al.* 1979) or vice versa (Rott *et al.* 1979). Thus, to re-state the major conclusions of these studies; (1) the HA must be cleavable in a broad range of cells; (2) no single gene is responsible for pathogenicity and (3) in each reassortant, an optimal gene constellation is selected in Nature that allows survival in Nature and determines virulence.

Studies with the mammalian reoviruses have also implicated multiple genes in virulence. From these studies my colleagues and I found that the laboratory strains type 1 (Lang) type 2 (Jones) and type 3 (Dearing) differed considerably in their overall patterns of infection. We thus isolated a number of reassortants and studied their pathogenesis. Interestingly, we found that each of the components of the viral outer capsid played a distinct role in virulence (Fields 1982). These findings with each component ($\sigma 1$, $\mu 1C$, $\sigma 3$) are summarized below.

Role of the S1 dsRNA segment ($\sigma 1$ protein): the viral haemagglutinin (table 1)

After entering the mammalian host, reoviruses are associated with lymphoid tissues and, as with most other infectious agents, induce an immune response. Two types of antibody activities are generally detected: neutralization antibodies and haemagglutination-inhibiting antibodies. Using intertypic recombinants, we were able to show that the $\sigma 1$ polypeptide is the type-specific antigen, responsible for determining specificity in both neutralization (NT) and haemagglutination inhibition (HI) (Weiner & Fields 1977; Weiner *et al.* 1978). To determine whether

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both types of antibodies react to the same site on the σ1 polypeptide, a number of monoclonal antibodies have been isolated that are directed against the σ1 polypeptide (Burstin *et al.* 1982). Interestingly, at least three antigenically active sites (epitopes) have been identified: one acts directly at a major site involved in neutralization (NT), a second site blocks haemagglutination inhibition (HI); and a third reacts both with NT and HI antibody. Thus the properties of NT and HI, although both presumably mediated by binding of the σ1 protein to cell surfaces, are properties of different regions of the HA molecule.

TABLE 1. ROLE OF THE S1, M2 AND S4 dsRNA SEGMENTS IN VIRUS–HOST INTERACTIONS

S1	1.	*cell and tissue tropism*
		(*a*) nervous system
		type 1, ependyma
		type 3, neurons
		(*b*) pituitary
		type 1, anterior
		type 3, intermediate and posterior
	2.	*specificity of host immune response*
		(*a*) humoral
		neutralizing antibody
		haemagglutination inhibition antibody
		(*b*) cellular
		cytolytic T lymphocytes (Tc)
		T cell-dependent delayed-type hypersensitivity (T_{DTH})
		suppressor T cells and tolerance (Ts)
	3.	*interaction with cells*
		(*a*) binding to cellular microtubules
		(*b*) binding to lymphocytes and to cell membranes
		(*c*) inhibition of cellular DNA synthesis
M2	1.	response to proteases
	2.	capacity to grow in intestinal tissue
	3.	induction of tolerance after peroral inoculation
	4.	virulence within a serotype
S4	1.	inhibition of protein and RNA synthesis
	2.	binding to dsRNA
	3.	frequent mutations in high-passage stocks and defective interfering stocks
	4.	possible role in establishing persistent infection

In addition to humoral immunity derived from bone marrow-derived (B) cells, thymus-derived (T) cells are involved in the host immune response to the reoviruses. After inoculation of mice with reovirus, three types of T cells have been detected. We were initially able to demonstrate the appearance of cytolytic T lymphocytes (Tc) from spleens infected 1 week previously by intraperitoneal inoculation (Finberg *et al.* 1979). Subsequently, using a footpad swelling assay, we were able to show that 7 days after immunization with reovirus, T cells mediating delayed-type hypersensitivity (T_{DTH}) to reovirus are detected in lymph node tissues (Weiner *et al.* 1980). In addition, when u.v.-inactivated virus is given intravenously, a lack of DTH reactivity (tolerance) ensues; this is due to active suppression involving the generation of suppressor T cells (Ts) (Greene & Weiner 1980). In each of these cases (Tc, T_{DTH}, Ts) the reactions are serotype-specific and, by the use of recombinant viral clones, have been shown to be specific to the viral haemagglutinin. Although structural studies comparing the type 1, 2 and 3 HA molecules have clearly shown that there are unique and shared sequences (Gentsch & Fields 1981), we have not yet directly identified the biochemical sites responsible for the various functions of the HA.

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To determine which viral gene is responsible for cell and tissue tropism, we took advantage of the differing tropism for cells in the nervous system exhibited by the different serotypes of reovirus. After inoculation of suckling mice with type 3 reovirus, a highly lethal encephalitis develops that is accompanied by destruction of neuronal cells without damage to ependymal cells. Type 1 infection results in a non-fatal infection involving ependymal cells that line the ventricular cavities of the brain, with little or no effect on neurons. Reassortant viruses containing nine genes from type 1 and S1 gene from type 3 (1. HA 3) produce a fatal encephalitis with neuronal destruction, whereas reassortants containing nine genes from type 3 and the S1 gene from type 1 (3. HA 1) produce a non-fatal ependymal infection (Weiner *et al.* 1977, 1980). Immunofluorescent studies indicate that the viral antigen is in ependymal cells in animals infected with reoviruses containing an S1 of type 1 and is in neurons in animals infected with reoviruses containing an S1 of type 3. Thus the S1 gene encoding the viral haemagglutinin is the determinant of cell tropism in the central nervous system (c.n.s.).

Studies of Notkins and colleagues (Onodera 1981) have confirmed the role of the S1 in determining c.n.s. cell tropism. In addition, they have found that the reovirus haemagglutinin determines cell tropism in the pituitary: the type 1 S1 leads to anterior pituitary infection whereas the type 3 S1 leads to sparing of the anterior pituitary and minimal involvement of the intermediate and posterior pituitary. Thus the viral haemagglutinin is responsible for cell tropism both in the c.n.s. and in the pituitary. It is therefore quite likely that viral haemagglutinin determines cell tropism in other sites as well.

All of the properties of the σ1 protein imply a role of the σ1 protein in recognizing and binding to receptors on the surface of the cells; the results imply that the σ1 protein is the protein responsible for attachment of reovirions to cells. Although direct data on the nature of cellular receptors for the reoviruses do not yet exist, studies have been performed indicating a role of the σ1 protein in attaching to or binding to cells or cellular components. By using reovirus particles as a probe for cell surface receptors it is possible to show that reovirus type 3 but not type 1 binds via the σ1 protein to the surface of murine and human lymphocytes (Weiner *et al.* 1980). In addition, cells exposed to the labelled extracts of reoviral infected cells contain only the σ1 protein (Lee *et al.* 1981). Joklik has suggested that, in L cells, reovirus particles of all three serotypes attach via the σ1 protein to the same cell-surface receptors (Lee *et al.* 1981). It is unlikely that the specific binding of different reovirus serotypes to the differentiated cell types that exist in host tissue will be to identical receptors. In addition to the occurrence of binding to cell surfaces, the σ1 protein mediates binding of reoviruses to cellular microtubules (Babiss *et al.* 1979). In these experiments the binding of reovirus type 1 or 3 to microtubules is measured *in vitro*. The higher degree of binding of type 1 enabled us to prove genetically that binding is mediated by the σ1 protein. The biological significance of this observation remains unclear.

Lastly, the σ1 protein mediates the inhibition of cellular DNA synthesis seen in cells infected with reovirus type 3 (Sharpe & Fields 1981). While the mechanism of this inhibition is not known, it is clear that it is not due simply to inhibition of protein synthesis because the inhibition of protein synthesis is a property of the S4 gene (see below).

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Role of the M2 dsRNA segment (μ1C protein) (table 1)

The upper alimentary tract is the natural portal of entry for reoviruses and other 'enteric' viruses. The fluids in the intestinal lumen as well as the material covering the gastrointestinal lining cells are the primary materials that reoviruses encounter after their introduction into the host. It is therefore not surprising that the nature of these fluids and host components play an important role in determining virulence after the peroral route of inoculation.

Differences between the behaviour of the type 3 Dearing strain and the type 1 Lang strain have allowed us to identify a critical role for the M2 dsRNA segment. Reovirus type 3 Dearing is highly neurovirulent after intracerebral (i.c.) inoculation (Margolis *et al.* 1971; Raine & Fields 1973; Weiner *et al.* 1977, 1980). However, after introduction of reovirus type 3 directly into the upper intestinal tract, it is avirulent (l.d.^{50} of more than 10^7) (Rubin & Fields 1980). The capacity of reovirus type 3 to grow in intestinal tissue is markedly impaired; after peroral (p.o.) inoculation there is a progressive loss of titre without significant growth. In addition, little or no virus reaches the c.n.s. and there is little viral growth in the brain. In contrast to these results with reovirus type 3 Dearing, the Lang strain of serotype 1 grows well in intestinal tissues. The capacity of reovirus type 1 to grow in intestinal tissue correlates with the efficient spread of virus from the gastrointestinal tract to the nervous system and growth of the type 1 in the c.n.s. The yield of reovirus type 1 in ependymal cells in the c.n.s. after following p.o. inoculation is thus similar to that after i.c. inoculation. To determine the genetic basis for this difference in behaviour, a number of reassortants were examined that were derived from type 1 Lang and type 3 Dearing (Rubin & Fields 1980). Reassortants that contain the M2 segment derived from type 1 behave like type 1; they grow in intestinal tissue and spread well to the nervous system. Reassortants containing the M2 segment derived from type 3 behaved like type 3; they do not grow in intestinal tissue and do not spread well to the nervous system. In fact, in contrast to the avirulence of the two parental viruses, reassortants containing an M2 derived from type 1 and S1 derived from type 3 are neurovirulent. The M2 gene of type 1 thus allows reovirus type 3 to grow and spread, whereas the S1 gene of type 3 causes the reassortants to enter neurons.

To determine the basis for these properties of the M2 gene, a number of studies were performed *in vitro*. Because intestinal fluids and absorptive cells contain a variety of proteases, we reasoned that the differences in response of the two isolates might be related to their responses to intestinal proteases. To test this possibility, we examined the effect of chymotrypsin on type 1 Lang and type 3 Dearing. After treatment of type 3 with chymotrypsin there is a marked loss in infectivity. In contrast, under comparable conditions, the infectivity of type 1 is either unaffected or slightly enhanced. Genetic studies with the reassortants noted above indicate that the M2 dsRNA segment is responsible for determining these differences in response to chymotrypsin (Rubin & Fields 1980). The fact that the digestion of reovirus *in vitro* by chymotrypsin mimics the pattern of growth of the serotypes and recombinants in the intestine as well as the spread to the c.n.s. suggests that chymotrypsin may be playing a role in determining the pattern of growth *in vivo*.

In addition to directly determining whether virus will grow in the gastrointestinal tract and then spread, the M2 gene plays a critical role in the development of host immunity after p.o. inoculation. We have found that, similarly to intravenous inoculation, p.o. administration of u.v.-inactivated reovirus type 1 Lang consistently leads to serotype-specific immunological

tolerance for delayed-type hypersensitivity responses to reovirus, that this unresponsiveness is secondary to the generation of Ts cells, and that these T cells are specific for the viral haemagglutinin (Rubin *et al.* 1981). Reovirus type 3 Dearing does not generate Ts cells after p.o. administration. This property is a function of the M2 RNA segment (Rubin *et al.* 1981). Thus, while the specificity of viral-induced tolerance resides in the S1 RNA segment (the viral haemagglutinin), the induction of tolerance is a property of the M2 segment.

Further insight into the role of M2 RNA in virulence has come from recent studies on the nature of relative virulence of isolates of the same serotype. A number of type 3 isolates were collected and, after preliminary studies on RNA (Hrdy *et al.* 1979), detailed analysis revealed a stable clone with reduced virulence after i.c. inoculation (Hrdy *et al.* 1982). To determine the basis for the avirulence of this clone, reassortants were prepared and a genetic analysis was performed. The property of avirulence resided entirely in the M2 RNA segment.

We still do not know the exact mechanism by which the M2 gene and the μ1C polypeptide affect virulence. It is possible that the effect is mediated by the interaction of the μ1C polypeptide in specific ways with host proteases that affect the capacity of the virus to undergo successful replication in infected cells (Rubin *et al.* 1981). In addition, direct inactivation of virus by host fluids and tissues may reduce viral titres below critical levels. Further studies should provide more insights into the detailed functions of this protein.

Role of the S4 dsRNA segment (σ3 protein)

Most of our studies with reoviruses have concentrated on reovirus types 1 and 3. Neither of these strains are very efficient at inhibiting host protein synthesis (Ensminger & Tamm 1969), although some inhibition is seen (Joklik 1974; Zweerink & Joklik 1970). When we began to study the properties of reovirus type 2, it became clear that type 2 is very efficient at inhibiting protein synthesis (Sharpe & Fields 1982) as reported previously (Loh & Soergel 1965, 1967). This provided us with an opportunity to determine which viral gene is responsible for inhibiting protein synthesis. A new set of reassortants were isolated between types 2 and 3 and, by analysing the different capacities of these strains to inhibit protein synthesis, we were able to show that the S4 RNA segment is responsible (Sharpe & Fields 1982). Although the precise mechanism is not clear, we also studied the effect of S4 on RNA synthesis and were able to demonstrate that the product of the type 2 S4 RNA segment inhibited RNA synthesis as well. Prior studies by Joklik and colleagues had shown that the σ3 protein specifically binds to dsRNA (Huismans & Joklik 1976). It remains to be determined whether this property and the effect on protein and RNA synthesis are related.

In addition to its effect on host cell metabolism, the S4 RNA segment has been found to be the site of frequent mutations. These are found both in virus that has been serially passaged at high levels (Ahmed *et al.* 1980) as well as in defective interfering stocks (Ahmed & Fields 1981). Furthermore, the S4 RNA segment is invariably derived from the DI virus during the establishment of persistent infection in L cells coinfected with T3-DI and wild-type 2 (Ahmed & Fields 1982). Whether there is an alteration in functions of S4 that plays a critical role during persistent infection remains to be determined.

It is thus clear that although these studies are in general agreement with the studies of influenza, each of the capsid proteins plays a distinctive role. Thus although certain 'gene combinations' may be more virulent than others, the most striking lesson of these studies is the different functions of the capsid proteins at each stage of infection.

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Recent studies with the bunyaviruses have begun to provide insights into the nature of bunyavirus infection of mosquitoes (Beatty *et al.* 1982). By using genetic approaches similar to those used on influenza and the reovirus, it was found that the middle-sized RNA segment (there are three RNA segments in the bunyaviruses), the segment that encodes the surface glycoprotein, is the major segment responsible for successful viral dissemination from infected midgut cells.

The possible role of mutation in affecting the results of pathogenesis studies with reassortant viruses

One of the striking findings in viral genetics over the last several years has been the extraordinarily high rate of mutation of animal viruses containing RNA genomes (reviewed by Holland *et al.* 1982). Since the goal of this review is to discuss the genetic basis of viral virulence, it seems worth while to discuss the possible role of such genetic instability on viral virulence as well as its possible role in complicating genetic analyses attempting to define the genetic basis of virulence.

The studies with the mammalian reoviruses noted above indicate that each of the genes encoding outer capsid polypeptides (S1, M2, S4) plays important roles in different facets of pathogenesis. It might be expected that mutations in each of these genes might alter the pattern of virulence in different ways. In fact, this is exactly what occurs (Fields & Greene 1982). The viral haemagglutinin is responsible for cell and tissue tropism. Mutations in critical regions of reovirus type 3 haemagglutinin are associated with attenuation and altered tropism in the c.n.s. The μ1C polypeptide determines sensitivity to chymotrypsin and growth in intestinal tissues. Mutations to resistance to digestion by chymotrypsin are associated with increased growth in intestinal tissues and increased lethality. Mutations in the S4 gene, the gene responsible for inhibiting cellular RNA and protein synthesis, are associated with persistent infection in cell culture. We have not determined the contributions of the S4 gene to animal pathogenesis. Thus it is quite apparent that viral mutations can dramatically alter the pathogenesis of viral infections, enhancing or reducing virulence.

How may mutations complicate analyses with reassortant viruses? A number of years ago we noted that temperature-sensitive (ts) mutants of reovirus type 3 contained altered μ1C polypeptides (Cross & Fields 1976), were altered in their response to certain proteases (Rubin & Fields 1980), and produced patterns of viral growth in intestinal tissues that were different from either 'wild-type parent' (Rubin & Fields 1980). We have subsequently discovered other examples indicating that when highly mutagenized ts viruses are used as parents for generating reassortants, intermediate phenotypes are often detected (see table 2). When the same properties are examined by using non-mutagenized parental viruses, intermediate phenotypes are either not present or are distinctly unusual. We interpret these results as indicating that silent, non-ts mutations have occurred at high frequency, and that such mutations are transferred into the reassortant progeny, resulting in phenotypes often intermediate between the two parental viruses (table 2). It is interesting that we have not detected such intermediate phenotypes in properties involving the S1 gene, whereas they are often present in M2 or S4. This may be due to the fact that functions of the S1 gene, by encoding the receptor interacting haemagglutinin protein, are qualitatively distinct (involving, for example, entry into distinct cell types) whereas properties of M2 or S4 are more quantitative in character. These studies thus indicate that when mutagenized parents, or even stocks passaged more than a few times,

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are used to generate reassortants, additional mutations present in the stocks can lead to intermediate results.

These results with the mammalian reoviruses suggested that some of the results with influenza reassortants may be due not simply to 'optimal gene constellations' but may represent multiple silent mutations present in the influenza strains used to generate the reassortants. Although it is likely, if not indeed certain, that some combinations of protein–protein interactions will lead to more stable or efficiently replicating reassortants, the possibility that silent mutations are influencing such experiments needs to be rigorously excluded.

TABLE 2. INSTANCES OF REASSORTANTS SHOWING INTERMEDIATE PHENOTYPES WHEN GENERATED FROM MUTAGENIZED PARENTAL VIRUSES

genome segment	phenotype	reference
M2	1. chymotrypsin sensitivity	Rubin & Fields (1980)
	2. growth in gastrointestinal tract	Rubin & Fields (1980)
	3. sensitivity to phenol	Drayna & Fields (1982)
S4	1. sensitivity to sodium dodecyl sulphate	Drayna & Fields (1982)
	2. inhibition of cellular RNA and protein synthesis	Sharpe & Fields (1982)

In summary, multiple viral genes clearly play a role in determining the pathogenesis of viral infections. By dissecting the various stages of pathogenesis it has been possible to demonstrate quite clearly this distinct role of several viral components. It is quite likely that discrete proteins play similar roles in infections caused by other viruses and diverse microorganisms.

We thank our colleagues for their contribution to this work. We acknowledge support of N.I.H. grants AI13178 and NS16998.

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