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Review

Experimental therapy of filovirus infections☆

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1. Introduction

The filoviruses (Marburg and Ebola) are perhaps the most mysterious of the viral agents that afflict humans. They emerge unpredictably in central Africa to cause epidemics of severe hemorrhagic fever, with high mortality rates (Sanchez et al., 2001; Bray, 2002). Their reservoir in nature is still unknown. The existence of filoviruses was first recognized in 1967, when workers in a vaccine production plant in Marburg, Germany were infected by contact with monkeys imported from Uganda. Since that time, Marburg virus has only been encountered in Africa. Ebola virus first came to notice in 1976 in an epidemic at a missionary hospital in Zaire (the present Democratic Republic of the Congo). Reported case fatality rates in that and other Ebola outbreaks have ranged from 60 to 90%, though many nonfatal infections have probably gone undetected. The Zaire, Sudan and Côte d'Ivoire subtypes of Ebola virus have only been found in Africa. By contrast, the fourth subtype, Ebola Reston virus, has only been isolated from macaques imported from the Philippines. Its relationship to the other filoviruses remains obscure. Ebola Reston causes severe, usually lethal illness in nonhuman primates. Limited circumstantial evidence suggests that it is less virulent for humans than the other viruses. All filoviruses are classified as biosafety level 4 (BSL-4) agents, requiring maximum biological containment.

Most filovirus outbreaks have apparently begun through the infection of a single individual from an unknown natural reservoir. Evolving epidemics have eventually become known to the outside world when progressive human-to-human spread has led to the death of doctors and nurses in a medical facility. Fortunately, experience has shown that filoviruses are not highly contagious and that prevention of direct contact with patients and their body fluids through barrier nursing procedures is sufficient to halt an epidemic. Although filoviruses are a source of fear in those areas of Africa where they occur, they are of minor importance on a global scale: fewer than

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2000 cases have been identified since the initial Marburg outbreak. However, the potential use of these viruses as agents of warfare or bioterrorism is of significant concern, as the occurrence of even a few Ebola or Marburg cases in a major city might cause panic and would certainly result in considerable economic expense.

No drug in clinical use provides significant protection against Marburg or Ebola infection. Current treatment is supportive in nature. Efforts to develop effective antiviral therapies have been handicapped by the limitation of research to a few laboratories and by the sporadic occurrence, remote location and brevity of outbreaks. This review aims to encourage contributions to the development of filovirus therapy, both by investigators working directly with these viruses and by others in the field of antiviral research. Investigative approaches not requiring BSL-4 containment are described in Section 5. The first half of this paper provides up-to-date information on filovirus biology and pathogenesis. The second half summarizes a variety of attempts to block individual steps in the filovirus replication cycle, both in vitro and in laboratory animals.

2. Taxonomy and genome organization

Filoviruses possess a single-stranded negative-sense RNA genome and thus, are members of the order Mononegavirales. Their name derives from the Latin *filum* (thread), reflecting their unique filamentous morphology. The family Filoviridae contains a single genus, *Filovirus*, made up of two species, the Ebola and Marburg viruses. The two species share a similar genomic organization, but differ significantly in nucleotide sequence and lack antigenic cross-reactivity (Sanchez et al., 2001). Ebola has four recognized subtypes (Zaire, Sudan, Reston and Côte d'Ivoire), while Marburg has none. Filovirus genomes are ≈ 19 kb in length. The linear arrangement of the seven viral genes resembles that of rhabdo- and paramyxoviruses. The 3′ and 5′ ends of the genome contain conserved, complementary sequences, which are important *cis*-acting regulators of genomic replication, transcription and possibly packaging. Transcription of individual genes is controlled by noncoding regions at the 3′ and 5′ ends, which contain short, conserved sequences.

3. Virion structure

Filovirus virions appear as long filamentous threads under the electron microscope. Viral particles vary in length from 800 to 14,000 nm, but have a constant diameter of 80 nm. The virion consists of a central ribonucleoprotein (RNP) core linked by two matrix proteins, VP24 and VP40, to a glycoprotein (GP)-bearing lipid bilayer derived from the host cell (Fig. 1A). The GP forms trimeric spikes on the virion surface (Fig. 1B); its molecular structure is described below. The RNP is comprised of the genomic RNA molecule and its encapsidating nucleoproteins, NP and VP30. Two other proteins present in lower copy number within the virion, VP35 and the RNA-dependent RNA polymerase, or L protein, associate with VP30 and NP to make up the

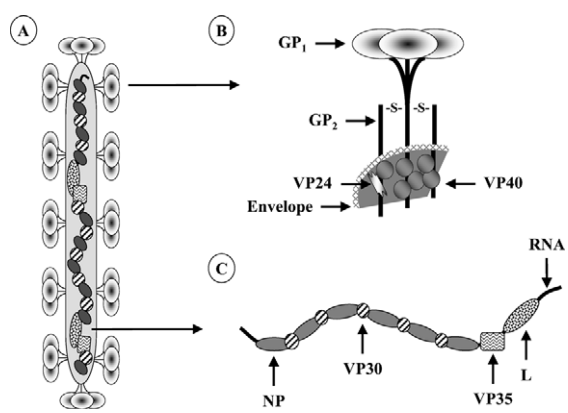

Fig. 1. Structure of a filovirus virion. See Section 3 for further information. (A) The ribonucleoprotein (RNP) complex in the virion core, made up of the RNA genome and its encapsidating proteins, VP30 and NP, is linked by matrix proteins to the glycoprotein (GP)-bearing lipid envelope derived from the host cell membrane. (B) Each molecule in the trimeric GP is composed of an external segment, GP_1, which is linked by a disulfide bond to a transmembrane unit, GP_2. The matrix proteins, VP24 and VP40, link GP_2 to the RNP. (C) The filovirus replication complex is made up of the genomic RNA molecule plus four proteins: NP, VP30, VP35 and the RNA-dependent RNA polymerase, or L protein.

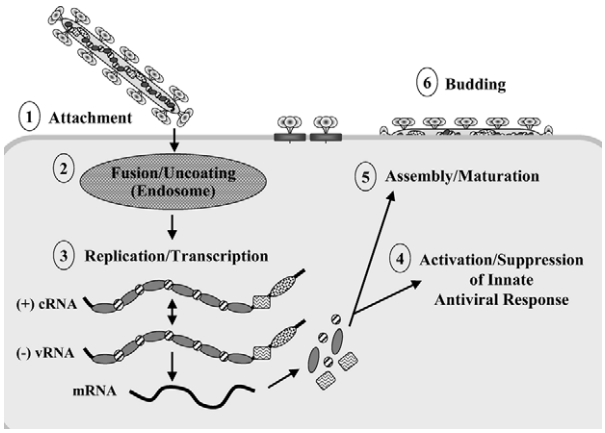

Fig. 2. The filovirus replication cycle. See Section 4 for additional information. Virions bind to a receptor on the cell surface (Step 1) and fuse with the cell membrane within endosomes (Step 2), releasing the RNP and other components of the replication complex into the cytoplasm, where transcription of viral genes and replication of the genome take place (Step 3). Viral replication triggers protein activation and a cascade of de novo protein synthesis that together make up the innate antiviral response (Step 4). Certain viral proteins appear to be capable of suppressing some components of this response. Virion assembly involves the movement of $GP_{1,2}$ to the cell membrane, the encapsidation of new genomic RNA molecules by NP and VP30 and the linking of the C-terminal cytoplasmic segment of $GP_{1,2}$ and the RNP by the matrix proteins VP24 and VP40 (Step 5). Nascent virions bud from the cell surface (Step 6).

Ebola replication complex (Fig. 1C) (Muhlberger et al., 1999). VP30 does not appear to be required for Marburg virus replication.

4. Replication strategy

A number of steps in the filovirus replication pathway offer targets for antiviral therapy (Table 1). The cycle resembles that of the better known rhabdo- and paramyxoviruses. Replication begins with the attachment of the virion to a cell-surface receptor (Fig. 2, Step 1), which may be the α-folate receptor (Chan et al., 2001). After binding, the virus enters the cell through receptor-mediated endocytosis (Geisbert and Jahrling, 1995). Fusion of the viral envelope with the cellular endosomal membrane releases the RNP into the cytoplasm (Fig. 2, Step 2). Transcription by the viral RNA-dependent RNA polymerase initiates at the 3′ end of the genome, resulting in synthesis of a leader RNA and seven polyadenylated mRNAs (Fig. 2, Step 3) (Sanchez et al., 2001). Accumulation of the first two proteins encoded at the 3′ end of the genome (NP and VP35) in some way triggers a switch to production of full-length, positive-sense 'antigenomes', which serve, in turn, as templates for genome synthesis.

In the case of Ebola, but not Marburg virus, the primary product encoded by the GP gene is a truncated protein lacking a C-terminal hydrophobic membrane anchor. This 'soluble GP' (sGP) is released from infected cells (Volchkov et al., 1998). Synthesis of full-length Ebola GP requires the insertion of an additional adenosine at an 'editing site' during the course of transcription (Volchkov et al., 1995). GP becomes abundantly glycosylated with *N*- and *O*-linked carbohydrates during its transit through the Golgi apparatus and is cleaved by a cellular furin-like enzyme in the trans Golgi to produce two units, an extracellular GP_1 and the transmembrane GP_2, which remain linked by a disulfide bond as $GP_{1,2}$ (Volchkov et al., 1998). The assembly of new viral particles occurs on the inner surface of the plasma membrane, where VP24 and VP40 apparently associate both with new RNPs and with the cytoplasmic tail of GP_2 (Fig. 1(B), Fig. 2, Step 5). Nascent virions become enveloped by the GP-bearing lipid bilayer and exit the cell through budding (Fig. 2, Step 6). The complete replication cycle takes $\approx$ 12 h.

As for many other viruses, filovirus replication triggers a cascade of reactions in infected cells, including the de novo production of type I interferon (IFN-α/β), which collectively constitute the innate antiviral response (Section 9) (Fig. 2, Step 4). At the same time, certain viral proteins apparently suppress some of these antiviral mechanisms, giving the virus an advantage over its host The ability of filoviruses to overcome initial defensive barriers and rapidly disseminate may be an essential factor in their virulence (Bray, 2001).

Table 1
Steps in the filovirus replication cycle (see Fig. 2), potential therapeutic countermeasures and published papers describing the in vitro or in vivo effect of each approach

Step	Process	Countermeasure	Therapeutic agent	Publications
1	Attachment to receptor	Neutralization of virus	Polyvalent antibodies	(Mikhailov et al., 1994; Jahrling et al., 1996; Markin et al., 1997; Cherpunov et al., 1998; Gupta et al., 2001a)
			Monoclonal antibodies	(Wilson et al., 2001; Maruyama et al., 1999a,b)
			Cyanovirin	–
		Blockade of receptor	–	(Chan et al., 2001)
2	Membrane fusion	Competitive inhibition	GP peptide analogs	(Watanabe et al., 2000)
3	Transcription and replication	Nucleoside analogs	Ribavirin	(Huggins, 1989; Ignat'ev et al., 1996; Markin et al., 1997)
		Impaired methylation of viral mRNA	SAH hydrolase inhibitors	(Huggins et al., 1995, 1999; Bray et al., 2000)
		Inactivation of viral genomic or mRNA	Antisense oligonucleotides	–
4	Suppression of innate antiviral response	Stimulation with IFN or IFN-inducers	IFN-α dsRNA (polyICLC) SAH hydrolase inhibitors CpG oligonucleotides	(Ignat'ev et al., 1996; Sergeev et al., 1997; Jahrling et al., 1999; Klinman et al., 1999; Bray, 2001; Bray et al., 2002)
5	Maturation and virion assembly	Interference with virion assembly and GP cleavage	–	(Harty et al., 2000, 2001)
6	Budding and release	Destruction of antigen-bearing cells	Antibodies	(Razumov et al., 1998)
			Immunotoxins	–
	Other	Undefined	Glycyrrhizic acid	(Pokrovskii et al., 1995)
	Physiologic derangements	Antidotes to: cytokine effects, coagulopathy, induction of apoptosis	Desferal, anti-TNF-α serum	(Ignat'ev et al., 1996, 2000)
			Coagulopathy inhibitors	–
			Apoptosis inhibitors	–

5. Surrogate viruses

The difficulties of working directly with filoviruses can be partially overcome by several different approaches. The first is the construction of recombinant 'pseudotyped viruses', which utilize the replicative machinery of vesicular stomatitis virus (VSV), murine leukemia virus or HIV-1, but package the Marburg or Ebola GP on the virion surface (Takada et al., 1997; Wool-Lewis and Bates, 1998; Kobinger et al., 2001). These chimeric viruses can be used at a low level of biological containment. They provide valuable information about such GP-related functions as virion binding, fusion and entry into target cells, but obviously cannot be used for studies of the intracellular stages of filovirus replication.

The latter processes can be studied using virus-free systems in which cells are transfected with plasmids encoding the individual protein components of the filovirus replication complex. Co-expression within the same cells of an RNA 'minigenome' containing a reporter gene flanked by filovirus 3′- and 5′-terminal genomic sequences permits the study of transcription and genomic replication (Muhlberger et al., 1999). This system could be used to screen compounds for inhibition of the filoviral RNA polymerase without the constraints of BSL-4 containment.

Another approach to identifying effective therapies for filovirus infections is based on the study of less virulent RNA viruses with similar replication strategies. Respiratory syncytial virus (RSV) is probably the most relevant 'surrogate virus' (Huggins et al., 1999). RSV causes severe respiratory tract infections in children of 'first world' countries, making it a more commercially viable target for the pharmaceutical industry than the rather obscure filoviruses. Recent developments in RSV therapy have been reviewed by Prince (2001). It should be noted that predictions of antiviral activity based on surrogate viruses are not always accurate. For example, although ribavirin is sufficiently active against RSV to be in clinical use for human therapy, it does not inhibit filovirus replication.

6. Pathogenesis of lethal infection

After gaining access to the body, filoviruses initially infect monocytes, macrophages and other cells of the mononuclear phagocytic system (MPS), probably in regional lymph nodes (Stroher et al., 2001). Some infected MPS cells migrate to other tissues, while virions released into the lymph or bloodstream infect fixed and mobile macrophages in the liver, spleen and other tissues throughout the body. Virions released from these MPS cells proceed to infect neighboring cells, including hepatocytes, adrenal cortical cells and fibroblasts.

Infected MPS cells become activated and release large quantities of cytokines and chemokines, including TNF-α, which increases the permeability of the endothelial lining of blood vessels (Feldmann et al., 1996; Villinger et al., 1999; Gupta et al., 2001a). Endothelial cells apparently become infected by virus only in the later stages of disease. Circulating cytokines contribute to the development of disseminated intravascular coagulation (DIC) by inducing expression of endothelial cell-surface adhesion and procoagulant molecules and tissue destruction results in the exposure of collagen in the lining of blood vessels and the release of tissue factor.

Massive lysis of lymphocytes occurs in the spleen, thymus and lymph nodes in the late stages of filovirus infection. There is no sign that the lymphocytes themselves are infected, rather they die through apoptosis, perhaps induced by cell-surface binding of chemical mediators released by MPS cells or by a viral protein (Geisbert et al., 2000; Hensley et al., 2002). Massive cytolysis, immune dysfunction, fluid shifts, microvascular coagulation and interstitial hemorrhage all play a role in the development of shock and death.

7. Clinical syndrome

Marburg and Ebola viruses cause similar diseases in humans, manifested as severe hemorrhagic fever. After an incubation period averaging ≈ 6 days, the onset of illness is abrupt, with headache, muscle pain, fever, nausea and vomit-

ing, abdominal pain and diarrhea. Conjunctival injection, pharyngitis and an erythematous macular rash may be present and are useful diagnostic signs. Abnormalities in standard laboratory tests include an early lymphopenia followed by neutrophilia, marked thrombocytopenia and prolongation of coagulation times, with circulating fibrin degradation products and other features of DIC. Serum levels of liver-associated enzymes, particularly aspartate aminotransferase, are elevated. Hemorrhagic phenomena generally take the form of petechiae, ecchymoses, conjunctival hemorrhages and oozing from venipuncture sites. Massive bleeding is rare. The onset of clinical deterioration is heralded by severe nausea and vomiting, prostration, tachypnea, anuria and a fall in body temperature. Death from shock usually occurs 6–9 days after the onset of symptoms.

8. Animal models

Three types of laboratory animals—mice, guinea pigs and nonhuman primates—are in use for testing antiviral drugs and vaccines and to study filovirus pathogenesis. The target cells of infection and the major pathologic features of fatal illness are similar in these diverse species. Nonhuman primates are exquisitely sensitive to all filoviruses, but guinea pigs and immunocompetent mice are inherently resistant to filovirus infection. This fundamental difference between animal models may result in divergent outcomes in tests of drug and vaccine efficacy.

8.1. Mice

Marburg and Ebola viruses cause fatal disease in newborn mice, but do not cause visible illness in adult immunocompetent mice (Bray, 2001). However, sequential passage of Ebola Zaire '76 virus in progressively older suckling mice resulted in the selection of a variant virus that causes rapidly lethal disease in normal adult mice when inoculated by the intraperitoneal route (Bray et al., 1998). The pathologic features of infection with this 'mouse-adapted virus' resemble those in primates, except that coagulopathy is much less prominent (Bray et al., 2001; Gibb et al., 2001). This mouse model is now in use for the preliminary testing of vaccines and antiviral drugs and for studies of filovirus pathogenesis. Immunodeficient mice, lacking either innate or antigen-specific immune responses, are susceptible to lethal infection by a variety of non-mouse-adapted Marburg and Ebola viruses. These murine models are proving to be a fruitful source of information on mechanisms of susceptibility and resistance to filovirus infection.

8.2. Guinea pigs

Guinea pigs develop a mild febrile illness after inoculation with Marburg virus or with Ebola Zaire or Sudan. Animal-to-animal transfer results in a progressive increase in virulence, resulting after a few passages in a viral stock that causes uniformly fatal disease. The major pathologic features of lethal infection in guinea pigs resemble those in mice and primates (Connolly et al., 1999; Ryabchikova et al., 1999). Guinea pigs have been employed for vaccine testing, but because of their size are less useful for the initial evaluation of experimental drugs, which tend to be available in only very small quantities.

8.3. Nonhuman primates

All filoviruses cause severe hemorrhagic fever in nonhuman primates. Ebola Zaire virus is the most virulent, producing uniformly lethal illness in African green monkeys, cynomolgus and rhesus macaques and baboons. In cynomolgus macaques, a commonly used model, this infection is characterized by the onset of fever and diminished activity on day 3–4 postchallenge; a reddish-purple macular rash on the trunk beginning on day 4–5; obtundation by day 6 and death on day 7–8. Virus is initially detectable in the serum on day 3 and titers may exceed 10^7 pfu/ml by day 5. High concentrations of virus are also measured in the liver, spleen and other tissues. Changes in blood cell counts and other clinical laboratory parameters resemble those in humans. Mild hemorrhagic phenomena are common, but profuse bleeding is rare. Ebola Sudan, Ebola Reston and

Marburg viruses also cause severe hemorrhagic fever in nonhuman primates, but with a more prolonged clinical course and somewhat $< 100\%$ mortality.

9. Filoviruses and the innate antiviral response

The initial defense against virus infection consists of a complex, coordinated cascade of responses within the infected cell, including the de novo synthesis of some proteins and the activation of other pre-existing proteins, that act in concert to block various steps in viral replication. This innate antiviral response includes the production of type I IFN, which binds to specific receptors on neighboring uninfected cells, setting off a similar train of reactions that produce an 'antiviral state' (Sen, 2001). Not unexpectedly, a number of viruses have evolved a variety of mechanisms to subvert these responses (Garcia-Sastre, 2001). Evidence is accumulating that filoviruses possess such mechanisms. Antiviral therapy that blocks suppression of innate antiviral responses might be a highly effective countermeasure to filovirus infection (see Section 11.6).

Interactions between filoviruses and innate immune responses can easily be studied in mice. As noted, normal adult mice do not become ill after inoculation of filoviruses that have not been 'adapted' to mice through sequential passage. By contrast, both knockout mice lacking a functional type I response and normal mice treated with antibodies to murine IFN-α/β resemble primates in their susceptibility to rapidly lethal infection after inoculation of non-mouse-adapted Ebola Zaire, Ebola Sudan and Marburg viruses (Bray, 2001). The mouse-adapted variant of Ebola Zaire virus appears to have been selected during serial passage for its ability to suppress type I IFN responses of mice. There is evidence that the mouse-adapted virus is attenuated for primates, suggesting that the same mutation(s) that enable it to kill mice impair its ability to block primate-specific innate antiviral responses (Bray et al., 2001).

The nature of the interaction between filoviruses and innate antiviral mechanisms is less well defined for primates. Both humans and nonhuman primates infected with Ebola Zaire virus have elevated serum levels of IFN-α during the symptomatic phase of illness (Villinger et al., 1999; Gupta et al., 2001a; Hensley et al., 2002). This IFN is presumably synthesized by infected macrophages. However, in vitro experiments employing primary human macrophages have given conflicting measurements of IFN-α production. Gupta et al. found that cells infected with Ebola Zaire virus released only trace amounts of IFN-α late in infection, but Hensley et al. observed an early, strong IFN-α response (Gupta et al., 2001a; Hensley et al., 2002). Evidence of filovirus-induced suppression of innate antiviral responses in primate cells has been reported by Harcourt et al. The authors reported that Ebola Zaire-infected human endothelial cells failed to respond to exogenous dsRNA by producing type I IFN or up-regulating MHC class I protein expression and were resistant to exogenous IFN-α (Harcourt et al., 1998, 1999). Others have found that the Ebola Zaire VP35 protein has a function analogous to that of the NS1 protein of influenza virus, preventing the induction of type I IFN in infected cells (Basler et al., 2000). VP35 may act by binding to double-stranded RNA (dsRNA), preventing the activation of dsRNA-associated phosphokinase (PKR) and possibly other antiviral proteins.

10. Experience with human therapy

There are few published reports of treatment of human filovirus cases. Convalescent serum from surviving patients has been used in a few instances, but its efficacy has never been convincingly demonstrated. One individual accidentally inoculated with Ebola Sudan virus in the laboratory survived infection after treatment with both convalescent serum and IFN-α, but it is not clear that either form of therapy was responsible for the favorable outcome (Emond et al., 1977). A Russian laboratory worker accidentally infected with Marburg virus also survived infection (Nikiforov et al., 1994). Because the disease was diagnosed after the onset of illness, he was not treated

with immune serum or IFN, but underwent several rounds of extracorporeal blood treatment with hemosorbents and dialysis. These measures produced transient improvements in his condition, but one cannot conclude that they caused his survival. Several Russian laboratory workers who were possibly exposed to Ebola Zaire virus in laboratory accidents have been treated with a preparation of equine anti-Ebola immunoglobulin and recombinant IFN (see below) (Kudoyarova-Zubavichene et al., 1999).

An attempt was made toward the end of the 1995 Ebola Zaire outbreak, to demonstrate the therapeutic efficacy of whole blood transfusions from convalescent survivors (Mupapa et al., 1999). The initial impression was highly favorable, since of eight laboratory-confirmed patients who received blood, only one died. The latter individual was treated 4 days after the onset of illness, while the seven survivors had already been sick for at least 11 days by the time they were transfused. Further analysis of data from that outbreak revealed that any patient who managed to live for 11 days after the onset of symptoms was almost certain to survive the infection, therefore the favorable outcome in these cases cannot be attributed to blood transfusion (Sadek et al., 1999).

11. Targets for antiviral therapy

Knowledge of the filovirus replication cycle has identified a number of targets for pharmacologic intervention. Given the extreme severity and rapid evolution of filovirus infections, it seems unlikely that any single therapeutic measure would be sufficiently potent to do more than slow the pace of disease progression. However, even a treatment that only prolongs the incubation period might be of benefit by providing additional time for the patient's immune system to mobilize against the infection. As discussed below, the most effective approach to filovirus therapy may turn out to be a combination of pharmacologic agents with different mechanisms of action.

11.1. Prevention of virion binding

Most attempts to neutralize circulating virus have involved the inoculation of infected animals with large doses of polyvalent antifilovirus antibodies. Such therapy has given variable results, depending on the source of antibodies and the animal model employed. Differences in outcome between the various animal models may result from the fact that mice and monkeys develop higher circulating viral titers and become ill more rapidly than guinea pigs and baboons, making it more difficult to neutralize all circulating virus. The efficacy of antibodies transferred between animal species is also affected by the kinetics of heterologous antibody clearance by the recipient's immune system and by incompatibility between the Fc portion of donor IgG and the recipient's Fc receptors (Wilson et al., 2001).

The most extensive experience with antibody therapy has been obtained using purified IgG from horses repeatedly inoculated with both inactivated and live Ebola Zaire virus (Krasnianskii et al., 1994; Borisevich et al., 1995; Krasnianskii et al., 1995). As noted, this material is now approved in Russia for human use in cases of laboratory-acquired infection (Kudoyarova-Zubavichene et al., 1999). Inoculation of 6 ml of this 'equine IgG' either 2 h before or immediately after viral challenge prevented the death of most baboons infected with Ebola Zaire virus (Mikhailov et al., 1994; Kudoyarova-Zubavichene et al., 1999). IgG isolated from sheep and goats hyperimmune to Ebola Zaire virus was also protective in baboons (Markin et al., 1997; Cherpunov et al., 1998; Kudoyarova-Zubavichene et al., 1999). Similar pre- or immediate post-exposure prophylaxis with equine IgG was also successful in Ebola Zaire-infected guinea pigs (Jahrling et al., 1996). However, large doses (up to 3 ml/kg) of equine IgG given within 1 h after viral challenge only delayed the onset of illness in mice and cynomolgus macaques by 1–2 days and did not prevent death (Jahrling et al., 1999). In another test of polyvalent antiserum, a high degree of protection was obtained in mice inoculated with pooled homologous serum from mice that had survived infection with mouse-adapted Ebola Zaire virus

(Gupta et al., 2001b). Treatment up to 2 days after challenge prevented the death of both immunocompetent and SCID mice.

Monoclonal antibodies (mabs) have also been tested as potential therapeutic agents. Murine mabs specific for Ebola Zaire GP prevented the death of Ebola-infected mice when inoculated up to 2 days after viral challenge (Wilson et al., 2000). The panel of antibodies reacted with five different epitopes on GP. Some were protective in vivo even though they did not neutralize the challenge virus in plaque-reduction assays. Human mabs that react strongly with the Ebola Zaire GP, sGP or NP have been produced from phage-display libraries constructed using mRNA from survivors of the 1995 Ebola Zaire outbreak (Maruyama et al., 1999a,b). Their therapeutic efficacy in infected animals has not been reported. In addition to neutralizing circulating virions, antibodies may also participate in cell-mediated lysis of infected cells (see Section 11.7).

A variation on the use of antibodies to neutralize circulating virus employs covalently-linked bispecific antibody complexes that bind both to virions and to a second target molecule. Nardin et al. (1998) prepared such 'heteropolymer' antibody complexes directed against the Marburg virus GP and the complement receptor-1 on human erythrocytes. Ideally, such a system would remove virions from the plasma by fastening them to the surface of red blood cells, which would then be cleared from the circulation by phagocytic cells in the spleen, liver and other tissues. In vitro tests showed that these heteropolymers bound both inactivated and live Marburg virus to erythrocytes. Their in vivo efficacy has not been reported. It is thus not known whether virions delivered in this manner to phagocytic cells would be capable of replicating.

Another approach to virus neutralization is based on the attachment of substances other than antibodies to the virion surface. For example, a protein produced by cyanobacteria, cyanovirin-N, binds to high-mannose oligosaccharides on gp120 of HIV-1 and inactivates the virus (Boyd et al., 1997). Cyanovirin also has limited, but measurable activity against Ebola Zaire virus in cell culture and in mice (Bray and O'Keefe, manuscript in preparation). Other substances with greater affinity for the specific oligosaccharides of Marburg and Ebola virus GP should be sought and tested for antiviral activity.

11.2. Blockade of the virus receptor

The human folate receptor-α was recently identified as a receptor for filovirus attachment (Chan et al., 2001). The investigators found that pseudotyped retroviruses bearing Marburg or Ebola GP on their surface were able to infect HeLa cells, but not the Jurkat line of T lymphocytes. When Jurkat cells were transfected with a HeLa expression library, only those cells that expressed the α-folate receptor permitted pseudotyped virus entry. Monoclonal antibodies to the folate-α receptor partially blocked infection. An earlier study identified the asialoglycoprotein as a receptor for Marburg virus (Becker et al., 1995). However, because this molecule is not expressed by a number of cell lines that are susceptible to filovirus infection, its role in pathogenesis is uncertain (Takada et al., 1997; Wool-Lewis and Bates, 1998). Identifying a specific filovirus receptor could potentially provide a target for small molecules that competitively inhibit virus binding.

11.3. Prevention of membrane fusion

Another approach to preventing infection of target cells is to block the release of the viral RNP into the cytoplasm by inhibiting virion fusion with the host cell membrane. This strategy is modeled after the 'T20' HIV-1 inhibitor, which reduced viral loads in AIDS patients (Kilby et al., 1998). Work with pseudotyped viruses has identified regions of the Ebola Zaire GP that interact with each other as part of the conformational change involved in fusion. An oligopeptide mimicking one of these sequences competitively inhibited viral entry (Watanabe et al., 2000). However, a concentration > 1 mg/ml was required to achieve 50% inhibition. It would be difficult to achieve such a level in the serum of a laboratory animal. HIV fusion inhibitors are effective at much lower concentrations, perhaps because retroviruses fuse with target cells at the cell membrane, while

filoviruses undergo fusion within endosomes. Improvements in targeting technology may be required to make fusion inhibitors a feasible approach for filovirus therapy.

11.4. Interference with transcription and genome replication

The viral RNA-dependent RNA polymerase is a major target of antiviral therapy. Although the nucleoside analog ribavirin inhibits the RNA polymerase of many RNA viruses, it does not block the transcription or replication of filoviruses in vitro or in vivo. Ribavirin treatment did not prevent the death of guinea pigs infected with Ebola Zaire, Ebola Sudan or Marburg virus or of monkeys infected with Ebola Zaire virus (Huggins, 1989; Ignat'ev et al., 2000). Similarly, administration of the related compound ribamidyl failed to protect Ebola Zaire-infected baboons (Markin et al., 1997; Cherpunov et al., 1998).

An alternative approach to blocking viral replication involves the use of antisense oligonucleotides that are complementary to sequences in viral genomic or mRNA. Such a strategy has been successfully employed to inhibit RSV replication (Torrence et al., 1997). In that case, the antisense oligonucleotide was covalently linked to a 2′–5′oligoadenylate sequence. Binding to the target activated cellular 2′–5′ oligoadenylate-dependent RNase L, resulting in cleavage of the RNA molecule (Adah et al., 2001). Experiments using this approach to block Ebola Zaire replication have so far failed to demonstrate sequence-specific inhibition (Bray and Torrence, unpublished data). Effective antisense therapy for filoviruses may require improved identification of critical regions of the viral genome that are accessible to oligonucleotide binding.

11.5. Inhibition of cellular S-adenosylhomocysteine hydrolase

A number of adenosine analogs, including the compounds carbocyclic 3-deazaadenosine (C-c^{3}Ado) and 3-deazaneplanocin A (c^3-Npc A), suppress the replication of a broad range of DNA and RNA viruses, including filoviruses (De Clercq, 1987, 1998; Huggins et al., 1999). These compounds inhibit a cellular enzyme, *S*-adenosylhomocysteine (SAH) hydrolase, which cleaves the SAH produced from *S*-adenosylmethionine (SAM) as a product of methylation reactions. Inhibition of SAH hydrolase increases the intracellular SAH/SAM ratio, resulting in feedback inhibition of methylation. The antiviral activity of adenosine analogs has been attributed to diminished methylation of the 5′ cap of viral mRNA by (guanine-7-)methyltransferase, which impairs the translation of viral transcripts (Cools and De Clercq, 1990; Oxenrider et al., 1993).

C-c^{3}Ado and c^3-Npc A strongly inhibit the replication of Ebola Zaire virus in vitro, with 50% inhibitory concentrations (IC_{50}) of 30 and 2 μM, respectively (Huggins et al., 1999). Initial testing in Ebola Zaire-infected SCID mice showed that three times daily treatment with either drug significantly reduced viral levels in tissues and prolonged survival, but did not prevent death (Huggins et al., 1995). Three times daily treatment of Ebola Sudan-infected African green monkeys with c^3-Npc A, begun on the day before infection, also prolonged their survival by several days (Huggins, unpublished data). A similar treatment regimen was much more effective in adult, immunocompetent mice infected with the mouse-adapted variant of Ebola Zaire virus (Huggins et al., 1999). All mice treated with low doses of C-c^{3}Ado or c^3-Npc A three times a day for 1 week or more survived infection, so long as treatment was begun on day -1, 0 or 1 postinfection. Therapy begun on day 2 or 3 was less protective.

Unexpectedly, it was found that even better results could be achieved by treating Ebola-infected mice only once, on day 0, 1 or 2, with a larger but nontoxic dose of c^3-Npc A or C-c^{3}Ado (Bray et al., 2000). Further investigation showed that the effect of treatment could be completely eliminated by co-administering antibodies to murine IFN-α/β, indicating a link between SAH hydrolase inhibitors and the type I IFN response (Bray, 2001). Additional studies have revealed that treatment with c^3-Npc A on day -2 or -1 is partially protective and that the drug causes massively increased production of IFN-α in infected, but not in uninfected mice (Bray et al.,

2002). The mechanism by which c^3-Npc A stimulates IFN-α production has not been determined. Since treatment on day −2 or −1 is partially protective, it is evident that the compound induces a long-lasting biochemical change in cells, perhaps preventing the methylation of a molecule that plays an essential role in virus-induced suppression of the type I IFN response. This previously unrecognized effect of SAH hydrolase inhibitors may help to explain their broad-spectrum antiviral activity. Unfortunately, initial testing of c^3-Npc A in Ebola-infected monkeys failed to reveal stimulation of IFN-α production. The identification of compounds that produce the same effect in filovirus-infected primates as that observed in mice is a major goal of current research.

11.6. Enhancement of innate antiviral mechanisms

If filoviruses are able to suppress the production of type I IFN as part of their replication strategy, it would seem that treatment with exogenous IFN-α should always be of significant benefit. However, the degree of efficacy in experimental animals has varied, depending on the species employed. In mice, the recombinant chimeric B/D form of human IFN-α is highly protective against Ebola Zaire infection, if begun no more than 2 days after viral challenge and continued for 5–7 days (Bray, manuscript in preparation). The IFN-inducer polyICLC is partially protective in the same model. Prophylactic administration of synthetic oligodeoxynucleotides containing unmethylated CpG motifs, which stimulate macrophages to release a range of inflammatory cytokines, including IFN-α, also prevent the death of Ebola-infected mice (Klinman et al., 1999). As noted, the potent effect of SAH hydrolase inhibitors in mice results from their induction of a strong IFN-α response.

The efficacy of exogenous IFN-α appears to be weaker in guinea pigs. Russian investigators used a model of Marburg virus infection to study the efficacy of the licensed pharmaceutical products Reaferon (IFN-α) and Ridostin (a mixture of single-stranded and dsRNA) (Sergeev et al., 1997). Treatment initiated before viral challenge delayed, but did not prevent death, while treatment begun 3 days postinfection had no effect on the course of illness. In another experiment, Marburg-infected guinea pigs were treated daily with homologous IFN-α, beginning on the day of challenge (Ignat'ev et al., 1996). Treatment failed to prolong the mean time to death, compared to a control group. The lack of efficacy of IFN in these experiments may have resulted from the use of heterologous IFN in the former case and from the use of too low a dose of homologous IFN in the latter.

The only reported test of IFN-α therapy in nonhuman primates showed only a limited protective effect. Four rhesus macaques were treated with high doses (2×10^7 IU/kg per day) of recombinant human IFN-α 2b, beginning immediately after challenge with Ebola Zaire virus and continued daily (Jahrling et al., 1999). This resulted in a 1–2 day delay in the onset of illness, viremia and death, compared to two placebo-treated control animals. It is possible that better results could have been obtained using a type of IFN-α more closely akin to the native IFN of macaques. There is clearly a need for further testing of IFNs and IFN-inducers in nonhuman primates, if only to improve our understanding of interactions between filoviruses and the type I IFN response.

If suppression of innate antiviral responses is part of the filovirus replicative strategy, it may be possible to pharmacologically prevent or reverse this effect. Such therapy might either completely block the development of infection or else slow its progression enough to permit the mobilization of antigen-specific immune responses. It is possible that the induction of a strong IFN-α response by SAH hydrolase inhibitors in Ebola-infected mice results from such a reversal of virus-induced suppression of innate antiviral responses.

11.7. Interference with viral maturation, assembly, budding and release

Pharmacologic blockade of a viral protease has proven to be a successful strategy for HIV therapy, particularly when combined with other forms of treatment. However, filoviruses do not encode their own protease that might serve as a target for

an inhibitor. The only proteolytic step in filovirus replication involves the cleavage of GP into $GP_{1,2}$ by a cellular furin-like protease (Volchkov et al., 1998). It has recently been shown that a genetically engineered Ebola Zaire virus lacking the cleavage site is able to replicate normally in cell culture (Neumann et al., 2002). This suggests that even if an inhibitor could block this step in maturation, it would have little or no effect on viral replication.

The interaction of GP and VP40 in the course of viral assembly may offer targets for antiviral attack. VP40 is capable of self-associating into closed extracellular vesicles in the absence of other viral proteins (Harty et al., 2000; Jasenosky et al., 2001; Timmins et al., 2001). Optimum vesicle formation required an intact PPxY motif and was facilitated by interaction with cellular ubiquitinating enzymes. It is possible that compounds could be found which block these interactions. For example, Harty et al. (2001) have demonstrated that certain proteosome inhibitors inhibit VSV replication. They found a correlation between the loss of ability of the VSV matrix protein to form extracellular vesicles in vitro and a reduction in replication of a viral culture treated with the same substances. This strategy might also be efficacious for filoviruses.

The expression of viral antigens in the cell membrane in the course of virus maturation may render the cell vulnerable to antibody-dependent, cell-mediated cytoxicity by natural killer cells and MPS cells. This mechanism may play a role in protection by antibodies, including non-neutralizing monoclonal antibodies to the Ebola Zaire GP. It is possible that additional virion components become accessible to immune recognition in the course of budding. Thus, it was observed that mabs specific for the Marburg virus VP40 protein did not neutralize the virus in vitro, but caused complement-mediated lysis of infected cells (Razumov et al., 1998). In a single reported experiment, these antibodies protected guinea pigs against lethal Marburg virus challenge.

Another method of attacking infected cells involves the use of toxins coupled to antibodies that recognize viral antigens on the cell surface. A number of reports have demonstrated that various cytotoxic compounds fused to mabs are capable of destroying virus-infected cells (Barnett et al., 1991; Berger et al., 1998; Pincus et al., 2001). The value of this approach may be limited by the wide tissue tropism of filoviruses, with consequent potential for severe tissue damage, particularly hepatotoxicity.

11.8. Modulation of the clinical syndrome

Successful treatment of a disseminated filovirus infection may require both an attack on viral replication and the administration of additional pharmacologic agents to mitigate the myriad physiologic effects of illness, including cytokine release and derangements of the coagulation system. Although much thought has been given to these questions, little published research has appeared. One exception is a report on the use of antiserum to TNF-α in the treatment of Marburg virus infection. When Marburg-infected guinea pigs were treated daily, beginning on day 3 postinfection, with anti-TNF-α serum, three out of five animals survived (Ignat'ev et al., 2000). Similarly, when Marburg-infected guinea pigs were treated with the compound desferoxamin (desferal), which allegedly blocks the induction by TNF-α of endothelial cell adhesion molecules, three out of six survived infection (Ignat'ev et al., 1996). Although these results suggest some protective activity, neither has been confirmed in additional published studies.

As noted, the large-scale death of lymphocytes in lymphoid tissues throughout the body is a major pathologic finding in lethal filovirus infections. Lymphocytolysis occurs in mice and guinea pigs infected with Ebola Zaire virus and in nonhuman primates lethally infected with Ebola or Marburg virus. These viruses do not replicate in lymphocytes, rather cell death occurs through apoptosis, which may be caused by mediators released by infected MPS cells or by a viral protein (Geisbert et al., 2000; Hensley et al., 2002). Compounds capable of blocking the induction of apoptosis could contribute to recovery from filovirus infection by preventing the loss of immune function.

11.9. Antiviral agents with undefined mechanisms of action

Glycyrrhizic acid (GA) is a natural component of licorice extract that inhibits the replication of a range of DNA and RNA viruses, including retroviruses. There is evidence that GA and related substances inhibit membrane penetration and uncoating (Mayaux et al., 1994) and also that they induce IFN-α production (Abe et al., 1982). GA and the related compounds glycyram and niglisin inhibited Marburg virus replication in vitro, apparently by blocking virus spread to uninfected cells (Pokrovskii et al., 1995). Large, twice-daily doses of glycyrrhizic acid were partially protective in Ebola-infected mice, delaying the onset of illness and death (Bray and Pokrovskii, unpublished data).

12. Promising avenues of research

A successful attack on the filoviruses will probably require improved understanding of the molecular mechanisms responsible for their extreme virulence. The chances of obtaining such insights have recently increased due to success in producing recombinant Ebola Zaire virus from a full-length cDNA clone (Volchkov et al., 2001). As an example of the potential utility of this new technique, its developers produced a genetically engineered virus in which the 'editing site' in GP was altered to prevent the synthesis of sGP. The modified virus caused more rapid lysis of infected cells than its wild-type counterpart, but grew to lower titer in cell culture, suggesting that it might be less virulent in vivo than the wild-type virus. This 'reverse genetics' method could facilitate the development of effective therapies by clarifying which potential targets for intervention are worth pursuing. For example, it was recently demonstrated that a genetically engineered Ebola virus lacking the site for GP cleavage is able to replicate in cell culture, indicating that pharmacologic blockade of GP cleavage would not be of therapeutic benefit (Neumann et al., 2002).

Another rapidly developing area of study is the investigation of virus-encoded inhibitors of innate antiviral responses. A number of studies have shown that the expression of viral IFN-antagonist proteins is required for virus replication in animals or cells with normal innate immune function. For the filoviruses, some of these questions can be examined by using murine models to dissect the interactions between host factors and the replication of mouse-adapted or non-adapted viruses. In particular, it may soon be possible to confirm the proposed role of the Ebola Zaire VP35 protein as an IFN antagonist by modifying or replacing the VP35 gene of mouse-adapted Ebola Zaire virus (Basler et al., 2000). Such studies could lead to the development of potent countermeasures to viral replication, either by directly blocking the action of VP35 or by triggering the desired cellular response at a point downstream of the virus-induced blockade.

13. Prospects for human therapy

Those attempting to develop specific treatments for filovirus infections recognize two distinct situations. The first is the need for postexposure prophylaxis to deal with the accidental infection of a laboratory investigator or of a health care worker during an outbreak. In such cases, the goal of therapy would be either to prevent viral replication and dissemination or else to significantly slow its pace, providing time for antigen-specific immune responses to mobilize and suppress the virus. A number of approaches described above would be expected to have some degree of efficacy. Some are clearly much closer to human use than others. The most experience in experimental animals has been obtained with antibody therapy. It appears that anti-GP antibodies administered immediately after exposure could significantly slow the progression of infection in humans. Single large doses of heterologous antibody would be unlikely to harm patients not already immune to the foreign immunoglobulin. Experience with administration of anti-Ebola equine IgG has already been obtained in Russia.

For forms of therapy that are not filovirus-specific, the shortest path to clinical approval may be the demonstration of an additional indication for

a compound already in use against another viral agent. Thus, currently licensed forms of IFN-α in use for the treatment of hepatitis C may also be of value in retarding filovirus dissemination. IFN-inducers might also be of some benefit. The latter are not currently licensed in the US, but some are in use in Russia. Further experience with IFN therapy should be obtained in filovirus-infected nonhuman primates to support human testing. Despite their potent broad-spectrum antiviral activity, SAH hydrolase inhibitors have received only limited clinical evaluation because of problems with toxicity. Evidence of a potent protective effect in filovirus-infected nonhuman primates will be required before these compounds can be considered for human therapy. All of the other experimental approaches described above are still in the earliest stages of development and will require testing in rodent and nonhuman primate models before they can be considered for human therapy.

The second situation—treatment of patients who have progressed to symptomatic illness—is a far more daunting problem. Therapy for such cases is needed in order to reduce mortality in filovirus outbreaks, but in practice it will be very difficult to evaluate any experimental form of treatment under the conditions of an epidemic in Africa. Studies that meet standard requirements for the use of an investigational new drug will require informed consent, randomization to treatment and placebo groups, and careful collection of data in support of drug efficacy. Even if a properly conducted study can be initiated, it might not be possible to treat a sufficient number of patients to demonstrate a positive therapeutic effect before the institution of barrier nursing procedures brings the outbreak to a halt. Once the problems of testing a new therapy have been resolved, it may be found that no single form of treatment is sufficient to bring about the survival of a significantly increased percentage of Ebola or Marburg patients. Only a combination of agents with differing mechanisms of action, implemented as quickly as possible after diagnosis, may be capable of reducing the mortality rate in a filovirus outbreak.

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