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Review

Treatment of yellow fever

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Abstract

Yellow fever (YF) is a life-threatening mosquito-borne flaviviral hemorrhagic fever (VHF) characterized by severe hepatitis, renal failure, hemorrhage, and rapid terminal events with shock and multi-organ failure. A live, attenuated vaccine (YF 17D), in wide use for over 60 years, causes a disease identical to wild-type virus at an incidence of 2.5×10^{-6}. Our current understanding of the pathogenesis and treatment of YF (described in this brief review) is derived from studies of animal models (macaques, hamsters) that reproduce the features of human YF and from descriptive studies of human cases of naturally acquired and vaccine-associated VHF. The least understood, but potentially most important terminal events appear to be due to 'cytokine storm' and represent a potential target for therapeutic interventions. Areas for future study include dissection of cytokine-mediated events in animal models, the pathogenic role of the profound neutrophilia that occurs pre-terminally, the (pathological) role of adaptive immune clearance in pathogenesis, and treatments directed at cytokine storm. Antibody, interferon-α, polyICLC and other immune modulators are highly effective when administered before or within a narrow time window after infection, but are ineffective when given after the infection is established. A few antivirals have been evaluated (ribavirin, tiazofurin, carboxamide, pyrazoline compounds). Ribavirin has been used successfully to treat hamsters when the drug is given at high doses up to 2 days after virus infection (shortly before liver infection), but has not shown promise in nonhuman primate models. Future work should focus on evaluating higher doses of ribavirin alone or in combinations with potentially synergistic drugs, including interferons. Also specific inhibitors against other flaviviruses such as dengue virus should be investigated for potential pan–flavivirus activity since recent studies have shown that specific targets such as the flavivirus proteases and helicases are very similar in structure.

Keywords: Yellow fever; Antivirals; Interferon; Pathogenesis

Contents

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1. Yellow fever disease: the problem

Yellow fever (YF), a mosquito-borne flavivirus disease, is the prototypical viral hemorrhagic fever. YF has many features in common with other hemorrhagic fevers but is characterized by more severe liver injury. YF is only found in tropical South America and sub-Saharan Africa, where an enzootic transmission cycle involves tree-hole breeding mosquitoes and nonhuman primates. Between 1990 and 2004, 14,281 human cases were reported to the World Health Organization, of which 11,763 cases (82%) occurred in Africa, but there is significant underreporting.

After an incubation period of 3–6 days, the onset of illness is abrupt, with fever, chills, myalgia, lumbosacral pain, headache, conjunctival injection, viremia, and neutropenia (Monath et al., 2008). Over several days to a week, the disease progresses with appearance of severe asthenia, nausea, vomiting, epigastric pain, jaundice, renal failure, cardiovascular instability, shock and hemorrhage. Approximately 15% of person who become infected by mosquito bite, develop visceral disease with jaundice (Monath et al., 2008). Within this subset, case fatality rates range from 20% to 50%, for all cases or for hospitalized cases, respectively.

There is increasing evidence that the systemic inflammatory response syndrome (SIRS, 'cytokine storm') contributes to terminal events and death. The early signs of infection (listed above) are probably due to the innate immune response to infection, including interferon-α, TNF-α and other acute-phase reactants (APRs). Studies of toll-like receptor (TLR) activation in animals indicate that the virus is a broad agonist for multiple TLRs (2, 7 and 8) (Querec et al., 2006).

Yellow fever 17D vaccine, developed in 1936 and in wide use since the 1950s, is a live attenuated vaccine manufactured in hens' eggs. Vaccination results in a mild or subclinical infection and a transient, low-level viremia, which does not exceed 2 $\log_{10}$ plaque-forming units (PFU)/mL. In rare cases (overall incidence 1:200,000–300,000), the virus can invade the central nervous system and cause meningoencephalitis, which is usually self-limited; young age (<6 months), advanced age, and immune deficiency are risk factors for yellow fever vaccine-associated neurotropic disease (YEL-AND) (Monath et al., 2008). The vaccine virus retains neurovirulence for mice when inoculated intracerebrally, and the rarity of YEL-AND in humans is due to the low viremia and neuroinvasiveness of 17D virus in humans. More interesting and problematic is an acute viral hemorrhagic syndrome disease closely resembling wild-type YF, caused by an overwhelming infection with the 17D vaccine virus: yellow fever vaccine-associated viscerotropic disease (YEL-AVD) (Hayes, 2007). The incidence of YEL-AVD approximates 1:200,000, and the case fatality rate is 60%; advanced age and thymectomy are predisposing factors (Barwick, 2004; Monath et al., 2008; Hayes, 2007). Persons over 60 years appear to have ~16-fold higher relative risk of acquiring YEL-AVD compared to persons <60 years (Khromava et al., 2005). It is likely that a genetic defect in the innate immune response, possibly in the 2′,5′-oligoadenylate synthesis alleles, underlies the pathogenesis.

Both the continued occurrence of wild-type YF and of serious vaccine accidents caused by acute virus replication and injury underlie the need to develop a clearer understanding of the pathogenesis of YF and therapeutic interventions. The latter could be drugs focused on the virus, as a means of interrupting virus replication and therapies aimed at modulating the bystander effects of an overly exuberant host immune response. This paper will review what little is known about YF pathogenesis, and suggest avenues for future study and evaluation of interventions.

2. Animal models useful in the evaluation of treatment strategies

Although good animal models of human YF exist, studies to date have been largely descriptive. They are useful for study of antiviral drugs, but to date they have not been used to study an important pathophysiologic mechanism (cytokine storm/SIRS) that could be the target for treatment (Monath and Barrett, 2003).

Nonhuman primates (principally rhesus and cynomolgus macaques) develop a disease virtually indistinguishable from human YF after inoculation or mosquito infection with unadapted wild-type YF virus, except that the disease syndrome is foreshortened, with death occurring at the end of the first week (Tigertt et al., 1960; Monath et al., 1981). The pathogenesis and pathophysiology of YF in macaques, insofar as they are known, are described below.

Syrian hamsters (*Mesocricetus auratus*) develop hepatic dysfunction and necrosis resembling YF in humans, but only with mutant virus strains, e.g. Jimenez wild-type virus adapted by serial passage in hamster liver (Tesh et al., 2001; Xiao et al., 2001). These animals develop lethal infection characterized by high viremia, elevated aspartate aminotransferase (AST) > alanine aminotransferase (ALT), and bilirubinemia. In this model, virus appears in the liver on day 3 after intraperitoneal inoculation and peaks on days 4–5, with jaundice appearing on day 6, not unlike human YF (Julander et al., 2007a). Pathological changes resemble the human disease including hepatocellular necrosis with apoptosis [eosinophilic degeneration (councilman bodies)], microvesicular steatosis, and lymphoid hyperplasia in the spleen, with viral antigen present in affected tissues. The hamster model differs from human YF in the absence of a clear midzonal distribution of hepatocellular pathology, more severe lymphoid necrosis, and prominent pancreatitis. As in humans, there is a correlation between the severity of liver injury (as reflected by ALT levels, and lethality (Julander et al., 2007a). YF in the hamster model appears to be less virulent than in nonhuman primates. Hamster susceptibility is age dependent, so that it is possible to mimic the human case fatality rate of ~50%. This feature makes hamsters particularly useful for studies of antiviral drug treatment.

3. Pathogenesis

Very few patients with naturally acquired or YEL-AVD have been studied in detail. This section will attempt to synthesize data from animal studies using both wild-type and vaccine virus,

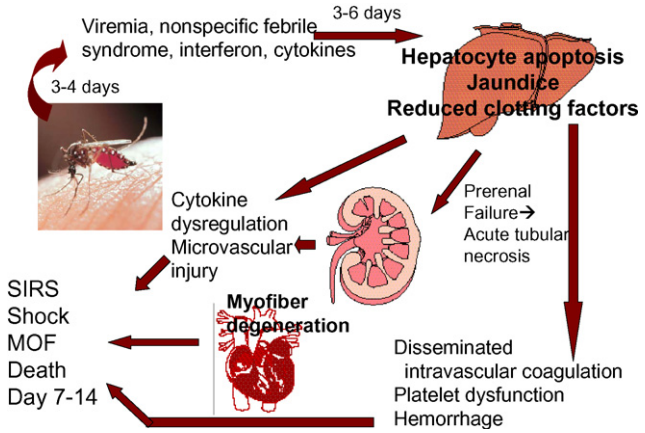

Fig. 1. Major events in the pathogenesis of yellow fever in humans and animal models. The likely sites of replication are the skin at the site of inoculation by the blood-feeding mosquito, the draining regional lymph nodes, mesenteric lymph nodes, spleen, resident macrophages in liver and other tissues.

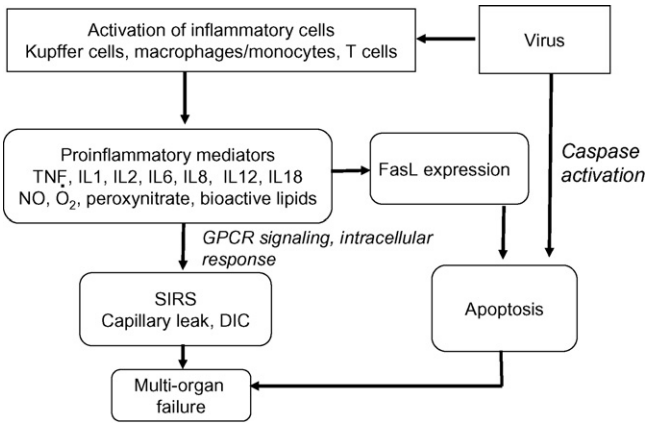

Fig. 2. Cell injury to liver and lymphoid tissues in YF is mediated principally by apoptosis. The systemic immune response syndrome is likely the final pathway to multi-organ failure, but is remains an area for future research.

and a small number of human cases with viscerotropic disease [see also a recent review (Monath and Barrett, 2003)].

After inoculation of the skin (by the bite of a blood feeding mosquito) or syringe/needle inoculation, virus replication occurs in draining lymph nodes, and then, with the ensuing viremia, in other tissues (Fig. 1). Immature and mature DCs are highly susceptible to YF virus infection, and unlike dengue, this is not dependent on DC-SIGN receptors (Barba-Spaeth et al., 2005). The biodistribution of YF 17D virus in monkeys revealed the predominant replication in lymphoid tissues that extended beyond the appearance of neutralizing antibodies (Monath et al., 2006). The sequence of infection after wild-type virus infection has not been studied, but it is known that infection of Kupffer cells, which guard hepatic sinusoids, precedes infection of hepatocytes (Tigertt et al., 1960). The route of hepatocyte infection is therefore presumed to be via Kupffer cells rather than direct viral access to the Space of Diss via sinusoidal fenestrations. Lymphoid tissues undergo profound changes in YF infection, characterized by appearance of large mononuclear or histiocytic cells, distension of the follicles, and necrosis of B cell germinal centers (Monath et al., 1981). It is presumed that the activation of cells in these tissues contributes to the systemic terminal features of YF, characterized by release of pro-inflammatory cytokines.

The liver is the most important organ affected in YF. Pathological changes observed in moribund animals and fatal human cases include eosinophilic degeneration of hepatocytes and Kupffer cells, and microvesicular fatty change. These changes are most prominent in the midzonal region and are due to apoptosis (Lefeuvre et al., 2006; Quaresma et al., 2006). Inflammatory cell infiltration is minimal, and characterized by Kupffer cells, NK cells, dendritic cells, CD4+, CD8+, and activated CD45RO+ T cells in the midzone and periportal areas (Fig. 1) (Quaresma et al., 2006, 2007). Expression of cytokines, including TNF-α, interferon-γ, and TGF-β is also localized in these areas of the liver lobule.

The terminal events occur precipitously and are characterized clinically by cardiovascular shock and multi-organ failure. The features of this phase strongly suggest that they are mediated by an inflammatory cascade (Fig. 2), although few patients have been directly studied. In one series of fatal and non-fatal naturally acquired disease, pro- and anti-inflammatory cytokines (IL6, IL8, TNF-α, monocyte chemoattractant protein-1, IL1-receptor antagonist, IL10), resembling bacterial sepsis were significantly elevated in fatal cases (ter Meulen et al., 2004), and similar findings were reported in a fatal case of YEL-AVD (Doblas et al., 2006). An overlooked feature of YF is the marked granulocytic leukocytosis observed in the terminal stage of disease. Given the elevated serum levels of IL-8 and TNF-α, it is likely that these granulocytes are activated, with consequent release of platelet activating factor (PAF), elastase and other proteases, and leukotrienes, which may modify endothelial integrity, particularly in the presence of pro-inflammatory cytokines (Takahashi et al., 2007), causing capillary leak. The syndrome in YF thus resembles many elements seen in overwhelming sepsis.

4. Does the adaptive immune response contribute to pathogenesis?

Many observations in animal models and human patients suggest that immune clearance by antibodies and T cells may contribute to pathogenesis and cytokine storm (Fig. 3). Co-circulating YF antigen (in the absence of detectable infectivity) and antibody, has been found in humans and monkeys, indicating the presence of immune complexes. During the terminal 24 h in the lethal rhesus monkey model, a rapid and acute decrease in antibody titer also reflects removal of virus-antibody complexes (Tigertt et al., 1960). This phenomenon coincides with multi-organ failure and shock. In a patient with fatal YEL-AVD, high-titers of neutralizing antibodies were found on day 8 coincident with a high virus load by PCR (Doblas et al., 2006). Antibodies could also mediate complement dependent cell killing and antibody-dependent cell mediated cytotoxicity (ADCC) targeting monocyte/macrophages expressing YF antigens. Clearance of infected cells by cytotoxic T lymphocytes would also contribute. These events would

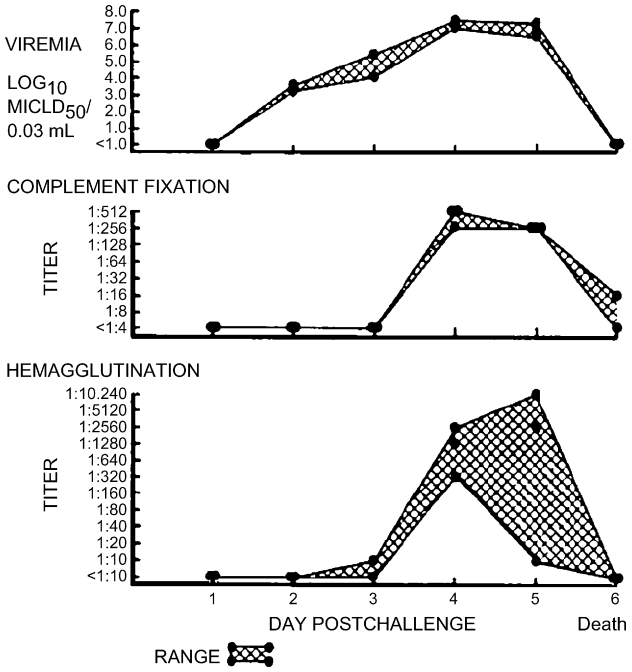

Fig. 3. The role of immunopathology in YF is presently uncertain, but the severe (terminal) events coincide with the onset of adaptive immunity, and adaptive immune clearance may precipitate cytokine-mediated oxidative stress, DIC, and capillary leak. Illustrated are data from YF infected macaques showing terminal decline in antibody titers indicative of immune clearance (from (Chu and Yang, 2007) ref. 5, with permission).

result in the release of pro-inflammatory cytokines, GPCR signaling, NFkB activation, and production of oxygen free radicals.

5. Treatment

Patients may benefit from intensive care [(modalities described in (Monath, 1987)], but despite the benefit of modern hospitals, the case fatality rate among patients with YEL-AVD and among a few travelers who became ill on return to the US or Europe has been 50–100%, suggesting that intensive care made little difference to outcome of this fulminating disease. There is no currently approved antiviral drug against YF, although it is likely that antiviral treatment, if developed, would be effective if given early in the disease.

5.1. Passive antibody

Antiserum to yellow fever produced in horses, monkeys, or chimpanzees protected rhesus monkeys against lethal yellow fever when given 1–3 days after lethal YF virus challenge (Pettit et al., 1928; Pettit, 1931). However, administration of immune serum or by cross-circulation from an immune donor monkey had no therapeutic effect in animals that had already experienced onset of clinical disease (US Army Medical Research Institute of Infectious Diseases, 1972). There is little clinical experience in passive immunotherapy; in one case, mouse monoclonal neutralizing antibody was given as a 'last resort' to a yellow fever patient in late stage hepatorenal failure without any beneficial effect (Colebunders et al., 2002). Based on available information, it is unlikely that antibody (like interferon or interferon inducers) would be useful except when given before onset of clinical disease or as treatment given during the earliest stages of illness. Further investigations are, nevertheless warranted using high-titer monoclonal antibodies. The potential immunopathological response to antibody treatment should be carefully evaluated.

5.2. Interferon and immunomodulators

The hamster model was employed to evaluate treatment with interferon-α administered IP at a dose of 0.5, 1.6 or 5 μg (kg day)$^{-1}$ starting 4 h prior to challenge and continuing to day 6 (Julander et al., 2007a). Whereas 40% of sham-treated controls died, survival was 80–90% in treated groups and there was a significant reduction in ALT levels. Interferon-α (5 mg (kg day)$^{-1}$ initiated 2 days after challenge also increased survival (90%) but animals had moderate hepatic dysfunction (Table 1).

African green monkeys (AGM) were infected with the Dak1279 strain of YF virus, which induces non-lethal infection characterized by viremia and hepatic dysfunction (Kende, unpublished). Treatment with recombinant human interferon-α (5×10^5 IU/kg) on days 1–7 resulted in complete suppression of viremia and a significant reduction in ALT levels (Table 1). However, one of four monkeys died late (25 days) and had liver lesions consistent with YF. Cynomolgus macaques given YF (Asibi) virus also sustained non-lethal infections, but with higher viremias than seen in AGM. In this model interferon-α 10^6 IU/kg by the subcutaneous route daily from day −1 to day 8) markedly reduced viremia and ALT levels compared to untreated animals.

In a separate study, AGM were infected with 800 pfu of YF (Asibi), and treatment with intravenous interferon-α (5×10^5 IU/kg) was given beginning on day 3 and continued to day 7. There was no effect on the level of viremia compared to sham treated animals (Kende, unpublished). These studies emphasize the need to treat with immunomodulators before or very shortly after infection.

Polyribocytidylic acid complex [poly (I) poly (C) linked to poly-L-lysine and carboxymethylcellulose] is a stabilized formulation that induced interferon-a via TLR3 activation. Poly-ICLC (3 mg/kg intravenously) was used to treat rhesus macaques 8 h before, 8 h or 24 h after challenge with YF (Asibi) at a dose of 1000 plaque-forming units (pfu), approximately 1000 LD50. Treatments were continued (days 1–4, 7, 9, 11, 15 and 17). Monkeys treated at −8 or +8 h survived or had prolonged survival time and markedly reduced viremias (Table 1) (Stephen et al., 1977).

Aryl acridine (3,6-bis[2-piperidinoethoxy]acridine triHCl) is an immunomodulator developed by Lederle Corp. in the 1980s and shown to be effective in mice infected with the neurotropic flavivirus, Banzi (Kende et al., 1988). A study in squirrel monkeys was conducted in which groups of four animals received drug (25 mg/kg) orally beginning on day −1 or +1 after YF challenge and continuing every other day through day 7 (Tignor and Kende, unpublished). Animals that received drug on day −1, but

Table 1
Nonclinical testing of various antiviral treatments against yellow fever

Treatment	Dose, schedule	Species	Challenge	Survival		Other outcome measures	Reference
				Treated	Control		
Interferon-α (consensus, Infergen®)	0.5, 1.6 or 5 μg (kg day)$^{-1}$, −4 h to 6 days after challenge	Hamster	YF (adapted Jimenez strain)	8/10–10/10 (80–100%)	4/10 (40%)	Animals gained weight, had reduced ALT levels compared to controls	Gabrielsen et al. (1994)
–	5 μg (kg day)$^{-1}$, −4 h to 6 days after challenge	–	–	90%	40%	–	–
Interferon-α	5 × 10^5 IU/kg IV days 1–7	African green monkey	YF (Dak 1279)	3/4 (75%) (1/4 animals, late death)	0/4 (0%)	Controls had peak viremia of 6.3 log10 pfu/mL, whereas treated animals had no viremia; controls had 5 × elevation of ALT vs. no elevation in treated group	Kende (unpublished)
Interferon-α	5 × 10^5 IU/kg, day +3–7 after challenge	African green monkey	YF (Asibi)	3/3 (100%)	3/3 (100%)	No difference in viremia profile between treated and control group	Kende (unpublished)
PolyICLC	3 mg/kg IV −8 h and on days 1–4, 7, 9, 11, 15, and 17)	Rhesus monkey	YF (Asibi)	5/7 (71%)	0/5 (0%)	100-fold decrease in viremia in treated group	Lefeuvre et al. (2006)
–	3 mg/kg IV +8 h and on days 1–4, 7, 9, 11, 15, and 17)	–	–	3/4(75%)	0/5 (100%)	100-fold decrease in viremia in treated group	Lefeuvre et al. (2006)
Aryl acridine	25 mg/kg PO day −1 to +7	Squirrel monkey	YF (?strain)	4/4 (100%)	4/4 (100%)	No viremia in treated group, 4/4 controls developed high viremia	Tignor, Kende (unpublished)
	25 mg/kg PO day +1 to +7	–	–	–	–	Both groups, all animals developed similar viremia	–
R-837, quinolinamine	20 mg/kg PO loading, then 10 mg/kg QOD			4/4 (100%)	1/3 (33%)	Treated animals had no viremia, ALT elevation; controls had high viremia and ALT	–
Interferon-γ	10^6 units (0.5 mg) IV −24 h, then Days 1–4	Squirrel monkey	YF (Dak1279)	6/6 (100%)	6/6 (100%)	Treated monkeys had 1 log lower peak viremia, shorter viremia, reduced ALT vs. controls	Malinoski et al. (1990)
–	–	Rhesus monkey	–	3/5 (60%)	1/5 (20%)	Treated monkeys had 2 day delay in viremia onset, reduced ALT vs. controls	Malinoski et al. (1990)
Ribavirin	50 mg/kg/day, −4 h to day 6, BID, IP route	Hamster	YF (adapted Jimenez strain)	9/10 (90%)	4/10 (40%)	Reduced ALT	Gabrielsen et al. (1994)
–	50 mg/kg/day, −day +2–8, BID, IP route	–	–	10/10 (100%)	4/10 (40%)	Gained weight, reduced ALT	Gabrielsen et al. (1994)
–	80 mg/kg load followed by 50 mg/kg QD IP, initiated 24–120 h after infection	–	–	87–100% (groups of 8–18 animals)	8/14 (57%	Treated animals had normal ALT vs. elevated ALT in controls; no effect on viremia	Monath et al. (1981)
–	50 mg/kg IV load, then 5 mg/kg TID beginning 16 h or 73 h after challenge	Rhesus monkey	YF (Asibi) 250 pfu				Monath et al. (2008)
–	50 mg/kg IV load, then 30 mg/kg TID	–	“2.5 pfu	2/4 (50%)	0/4 (0%)	–	Monath et al. (2008)
–	30 mg/kg IV load followed by 10 mg/kg TID for 7 days	“	YF Dak 1279 1000 pfu			–	Monath et al. (2008)
	30 mg/Kg IM QD days +3–7	African green monkey	YF (Asibi)	3/3 (100%)	3/3 (100%)	No differences in viremia peak or duration	Kende (unpublished)
–	50 mg/kg day −3 to +8	Squirrel monkey	YF Dak 1279 1000 pfu				Monath et al. (2008)

not those that were treated after infection were protected against viremia.

A derivative of quinolinamine (R-837, 3-M Corp.), 1-isobutyl-1*H*-imidazo[4,5-*c*) quinoline-4-amine, is a potent interferon inducer active topically, orally, and by injection. The drug was also evaluated in cynomolgus macaques challenged with YF (Asibi) 1000 pfu. A loading dose of 20 mg/kg orally on day −1 was followed by every-other day dosing at 10 mg/kg. All three untreated monkeys developed high viremias (5–8 $\log_{10}$ pfu/mL), high ALT levels, and two animals died. None of four treated monkeys developed viremia, hepatic dysfunction or illness (Kende, unpublished).

Interferon-γ 0.5 mg (10^6 units)/kg was administered IV to squirrel and rhesus monkeys 24 h before and at daily intervals for 4 days after challenge with 700 pfu of YF Dak1279 virus. Treated rhesus monkeys had lower mortality (40%) than untreated controls (80%), delayed onset of viremia, and significantly lower ALT levels. Similarly squirrel monkeys (which do not develop lethal infections), had shorter and reduced peak viremia, and significantly reduced ALT levels (Arroyo et al., 1989). Overall, the results were less impressive than those seen following treatment with interferon-α or polyICLC.

5.2.1. Ribavirin

Ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide) is a purine nucleoside having broad-spectrum activity against a variety of DNA and RNA viruses (Tam et al., 2003). Ribavirin in combination with pegylated interferon-α is effective for the treatment of chronic infection with hepatitis C virus, a relative of flaviviruses. Intracellularly, ribavirin is phosphorylated to ribavirin monophosphate, diphosphate, and triphosphate. Red blood cells phosphorylate ribavirin, but lack phosphatase activity so that phosphorylated ribavirin reaches high levels over time, causing hemolytic anemia, which is reversible. This adverse effect is a consideration for chronic treatment, but is less so for the short term therapy of life-threatening infections, such as Lassa fever, Congo-Crimean HF, and Hantaan virus infection, in which intravenous treatment has been successful (McCormick et al., 1986). Taribavirin hydrochloride, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine (Viramidine®, Valeant Pharmaceutical International, Singapore) is a prodrug of ribavirin; accumulation in erythrocytes is lower than with Ribavirin treatment (Yeh et al., 2007). Both Ribavirin and viramidine have shown promise in the hamster YF model (10, 30). Julander et al. (2007a) administered 100 mg (kg day)$^{-1}$ viramidine or 50 mg (kg day)$^{-1}$ ribavirin, and challenged hamsters with the liver-adapted Jimenez strain (see Section 3). It should be noted that YF virus was given at low dose to effect a 60% mortality in the animals, a mortality ratio not unlike that observed in humans with severe infections. When treatment was initiated 4 h prior to challenge and continued (50 mg/kg in divided doses BID through day 6), there was 90% survival and a significant reduction in serum ALT levels. Lower doses (5 mg (kg day)$^{-1}$ were not effective in the hamster model. Viramidine (100 mg/kg BID from −4 h to 6 days after challenge) was as effective as ribavirin. When treatment with ribavirin was initiated 2 days after challenge (∼1 day before appearance of virus in liver tissue and 3 days before liver dysfunction as measured by elevation of ALT), all hamsters survived and escaped chemical hepatic dysfunction. Sbrana et al. (2004) found that treatment with ribavirin at a high loading dose (80 mg/kg) followed by daily doses of 40 mg/kg IP resulted in 100% survival (vs. 57% in controls) and that treatment could be initiated as late as 72 h after infection. Animals treated as late as 120 h after infection showed reduced mortality. ALT levels were normalized in treated hamsters. Interestingly there was little/no effect on viremia. These studies of ribavirin suggest that the drug (and viramidine) should be evaluated clinically. In addition, the combination of ribavirin and interferon-α, which have synergistic activity in hepatitis C, should be studied.

The central question for use of these antiviral therapies is whether they will be effective when administered later in the course of infection (especially after the onset of visceral disease) or whether patients can be identified for treatment during the early stage (period of infection) of YF. The latter depends on the availability of RT-PCR or antigen-detection ELISA diagnostic tests that can be applied at the point of care. A second significant issue (as pointed out below) is the dosage of ribavirin (or viramidine) that should be employed clinically, since flaviviruses are less susceptible to ribavirin than arenaviruses, bunyaviruses and hantaviruses. The dosage of ribavirin that has been used to treat human VHF (e.g. Lassa fever) has been an intravenous loading dose of 30 mg/kg followed by 15 mg/kg q6h for 4 days and 7.5 mg/kg q8h for 6 days (McCormick et al., 1986). Mean plasma concentrations of ribavirin following this treatment regime can be estimated from pharmacodynamic studies with high doses of oral drug to be in the range of 20 μM (5 μg/mL) (Roberts et al., 1987). The concentration of ribavirin effective in vitro (Vero cell culture) against arenaviruses and bunyaviruses is in the range of 25 μg/mL, whereas 10-fold higher concentrations (250 μg/mL) are required to suppress YF virus (Huggins et al., 1984). Since ribavirin accumulates intracellularly, tissue concentrations far exceed those in plasma, which accounts for the antiviral activity in YF infections in hamsters. Skeletal muscle and red blood cells are the principal sites of tissue distribution, but about 8% of total body ribavirin is distributed in liver (Ferrara et al., 1981).

Ribavirin was studied in rhesus macaques given a 50 mg/kg loading dose and then 5 mg/kg q8h beginning early (16 h) or late (73 h) after infection with 250 plaque-forming units, pfu [approximately 250 LD50 of YF (Asibi)] (Huggins et al., 1984; Huggins, 1989). There was no effect on the lethal course of infection. A repeat study employed a higher dose (30 mg/kg q8h) and a reduction in the challenge dose (2.5 pfu), and in this case a modest effect was observed (50% mortality vs. 100% in untreated controls).

Another study in rhesus macaques was conducted by Huggins (1989), in which 16 animals were given Dak1279 virus (1000 pfu) and half of the monkeys were treated with ribavirin at 30 mg/kg loading dose, then 10 mg/kg TID for 7 days or placebo. No differences were observed across treatment groups with respect to survival or viremia level.

In a non-lethal model, AGM were infected with 800 pfu of YF (Asibi), and treatment with ribavirin 30 mg (kg day) by the

intramuscular (IM) route was given beginning on day 3 and continued to day 7. There was no effect on the level of viremia compared to sham-treated animals (Kende, unpublished). When ribavirin and interferon-α (5×10^5 IU/kg) were co-administered on days 3–7, viremia was slightly delayed (peak on day 4 instead of day 3) but the level was not diminished.

Unpublished studies were conducted in a squirrel monkey model by Greg Tignor and Robert Shope at Yale. The animals were infected with a high dose (1000 LD50) of the Dak1279 strain of YF virus. The animals were treated with ribavirin at 50 mg (kg day) beginning 3 days before and continuing for 8 days after challenge. Despite the high drug dose and pre-exposure initiation of therapy, the effect on survival and mean time to death was minimal and did not reach statistical significance.

A supporting study of prophylactic ribavirin in rhesus macaques infected with dengue type 1 virus was reported by Malinoski et al. (1990). Monkeys received a loading dose of 50 mg/kg IM followed by 10 mg/kg q8h for 10 days beginning on day −1. Plasma levels approached 30 μM (7.3 μg/mL). The principal outcome measure was viremia, since these animals develop no overt clinical signs. There was no treatment effect on the time course or height of viremia.

Thus, while ribavirin shows promise in a rodent model of YF, studies in nonhuman primates have not been promising, even when administered prophylactically, a setting that would have limited application in the field. The proviso is that the fine balance between dose and the severity of viral challenge may not have been optimal in these animal studies to show an effect. Thus, clinical studies are probably warranted, particularly in settings where treatment can be initiated early. Since ribavirin has shown promise in other infections where it is combined with interferon or interferon inducers, further nonclinical evaluation is also warranted. An important additional problem with the use of ribavirin may be its poor transport to the central nervous system. Since YF virus is neurotropic, treated patients who are rescued from hepatic infection might theoretically develop late encephalitic infections. This may be especially problematic in patients with neuroinvasion of YF 17D vaccine (in the setting of viscerotropic adverse events), since 17D is more neurotropic than wild-type virus.

5.3. Other antiviral drugs

A broadly active carboxamide drug [AT-1106 (2,4-dihydro-3-oxo-4-β-D-ribofuranosyl-2-pyrazinecarboxamide)] appears to have an acceptable safety profile and was effective in hamsters when treatment started after liver infection (day 4) with adapted YF virus (Julander et al., 2007b).

Other antivirals have not been so promising:

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide), a synthetic analogue of ribavirin is an inosine monophosphate dehydrogenase inhibitor, in late stage trials for treatment of leukemia. A study was conducted in three rhesus monkeys inoculated with a lethal dose of YF Dak1279 and treated with repeated high doses of tiazofurin (1100 mg/m^2). The drug was not effective (Kende, unpublished).

Orotidine 5′-monophosphate decarboxylase inhibitors, 6-azauridine and related compounds showed good in vitro inhibitory activity against YF virus (and another flavivirus, Japanese encephalitis, JE), but were not effective in a JE mouse model (Gabrielsen et al., 1994).

A number of isoquinolone alkaloid drugs extracted from Amaryllidaceae or synthesized showed good in vitro activity against YF virus, but one of the most active (pancratistatin, had marginal in vivo activity in the JE mouse model (Gabrielsen et al., 1992).

Iminocyclitol compounds with a deoxynojirimycin head group inhibit viral budding on the endoplasmic reticulum, and these molecules were active against flavi- and pestiviruses in vitro at micromolar concentrations (Gu et al., 2007).

Triaryl pyrazoline {[5-(4-chloro-phenyl)-3-thiophen-2-yl-4,5-dihydro-pyrazol-1-yl]-phenyl-methanone} inhibits flavivirus infection in cell culture, and has broad activity against other RNA viruses as well (Puig-Basaqoiti et al., 2006; Goodell et al., 2006). Another broad-spectrum agent capable of inhibiting flaviviruses is 2-amino-8-(beta-D-ribofuranosyl) imidazo [1,2-*a*]-*s*-triazine-4-one (ZX-2401); synergy with interferon was also demonstrated (Ojwang et al., 2005).

Future perspectives Pan–flavivirus drugs that target viral non-structural proteins.

Recent advances in structural biology and in the functional characterization of flavivirus proteins should yield dramatic break-through in the design of antiviral drugs. The crystal structures of the NS2B-NS3 protease complex and of the NS5 RNA polymerase have been defined, and critical amino acid residues identified underlying the functional domains of these proteins that play essential roles in replication (Erbel et al., 2006; Yap et al., 2007; Bera et al., 2007; Lin et al., 2007). High throughput assays for the functional activities of these proteins have been developed that will allow screening of inhibitor molecules. The key role of intra-nuclear localization of NS5 in replication, and of importin molecules in trafficking of NS5 from the cytoplasm to the nucleus and export back to the cytoplasm has identified a new target for antiviral drugs (Pryor et al., 2007). NS5 plays a central role in resistance to interferon by suppressing the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway. The specific amino acid residues responsible for this inhibition have been investigated, and represent a likely target for new antivirals (Park et al., 2007).

Recently the Novartis Institute for Tropical Diseases initiated a comprehensive program to find small molecule compounds that inhibit the replication of the four serotypes of dengue virus, by focusing the discovery efforts on the viral enzymes— the protease, helicase, methyl transferase and RNA-dependent RNA polymerase (Keller et al., 2006). Because the targeted viral activities are contained within two multifunctional non-structural proteins (NS3 and NS5) that are highly conserved amongst flaviviruses it is highly likely that anti-dengue compounds will be active against YF and related pathogens, including West Nile (WN) and Japanese encephalitis (JE) viruses. Indeed, a series of substrate based inhibitors that were optimized for the active dengue virus protease (Yin et al., 2006a,b) was found to show a similar activity profile to YF and WN virus (Knox et al., 2006)

suggesting that common inhibitors may be found for these targets. The similarities in X-ray structure of the helicase from dengue virus (Xu et al., 2005) and YF (Wu et al., 2005) are further indications that pan–flavivirus inhibitors can be found for this target as well.

5.3.1. Inhibition of host gene functions

An interesting approach to antiviral therapy is the inhibition of host cell enzymes as targets for antiviral therapy. The approach is based on the premise that host cell enzyme pathways, particularly kinases, have been usurped by viruses for various steps in entry and replication, and that, since these enzymes are redundant, it might be possible to inhibit a specific host pathway critical to virus replication without undue toxicity. Recently a c-Src kinase inhibitor (dasatinib) was shown to block maturation of flavivirus (Chu and Yang, 2007), indicating that may indeed be a promising approach to antiviral therapy.

5.3.2. Treatment of cytokine storm

An effective treatment for 'cytokine storm' associated with multi-organ failure would be useful in patients with YF, as well as many other viral hemorrhagic fevers and sepsis. Interestingly, a retrospective analysis of patients with YEL-AVD showed that use of stress dose corticosteroids was associated with significantly lower mortality (Vellozzi et al., 2006). More specific treatments targeting GPCR signaling [e.g. CXCR1/CXCR2 signal inhibition with pepducins (Kaneider et al., 2005)] or PAR1 signaling with activated protein C (drotrecogin alfa, Xigris®) in animal models are warranted. The hemorrhagic diathesis in YF might contraindicate inhibition of thrombin activation by drotrecogin alfa but experimental studies could be designed to study this question, and management of excessive bleeding with recombinant factor VIIa might be contemplated. As for antiviral drugs, the timing of initiation of treatment with GPCR inhibitors is likely to be critical, and early institution of treatment before inexorable multi-organ failure occurs is likely to be more effective.

6. Potential for clinical trials of antiviral strategies

Yellow fever cases occur annually, but generally in remote areas with rudimentary health services and no infrastructure for clinical research. Unlike Lassa fever, for which longitudinal studies of ribavirin treatment were possible in field research setting in a focus of recurring virus transmission (McCormick et al., 1986), it would be not be possible to find an appropriate site to study yellow fever cases. The most likely scenario in which a clinical trial could be performed is during an epidemic. Yellow fever epidemics have occurred at intervals in Africa, and the attack rate and duration (3–6 months) are sufficient for a study protocol to be approved and a study executed, although there should ideally be advanced logistical and regulatory planning. If a promising therapy is identified, compassionate use under an investigator IND in cases of YEL-AVD could also yield important information because of the inexorable course of the disease.

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