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Research on prevention and treatment of hemorrhagic fevers

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Abstract

Viral hemorrhagic fevers are severe zoonotic diseases caused by RNA-viruses classified into 4 families: *Arenaviridae*, *Bunyaviridae*, *Filoviridae*, and *Flaviviridae*. They are present on all continents except Antarctica, their person-to-person spread is easy, and there is a high risk of them being used as weapon by bioterrorists. So far, efforts to develop effective drugs against these viruses have failed, and typical therapy usually relies on symptomatic treatment. Search for substances that could effectively inhibit this type of infections is now a priority. The presented paper gives an overview of different approaches used in combating the viral hemorrhagic fevers. Researchers look for safe antiviral agents with appropriate properties among natural sources, such as various types of herbs plants, algae, or essential oils obtained from trees, as well as investigate the use of various synthetic substances. The aim is to broaden the pool of available antiviral drugs that could replace hitherto applied medicines such as ribavirin, which is not always sufficiently effective and may have side-effects. The scientists focus not only on combating the diseases, but also on their prevention. For this purpose, recombinant vaccines or various types of immunomodulators may serve as a useful tools. Results of the latest studies are promising and encourage further work which may eventually lead to the solution of the urgent problem of hemorrhagic fevers.

Key words

Hemorrhagic fevers, viruses, antivirals, vaccines

INTRODUCTION

Viral hemorrhagic fevers (VHFs) are zoonotic diseases caused by RNA-viruses belonging to 4 different families: *Filoviridae*, *Flaviviridae*, *Arenaviridae*, *Bunyaviridae*. The most important representatives are: Ebola (EBOV), Marburg (MARV), yellow fever (YFV), West Nile virus (WNV), dengue (DENV), Lassa (LASV), Junín (JUNV), Machupo (BHFV), Sabiá, Guanarito, Rift Valley fever virus (RVFV), Crimean Congo fever virus (CCHFV), hantaviruses, and the newly recognized Alkhumra virus, and others. Hemorrhagic fevers belong to the most severe infectious diseases. Typically, they are manifested by characteristic hemorrhagic diathesis (bleedings from body cavities and internal organs, or subcutaneous bleedings) that may have complex pathogenesis (damage to blood vessels, thrombocytopenia, and disseminated intravascular clotting – DIC). The mortality rate for some EBOVs can be up to 50-90% [1]. By May 2011, the Centers for Disease Control and Prevention (CDC) had recorded about 2,300 cases of Ebola virus infections, including 1,957 deaths – mortality rate of approx. 85% [2], and by November 2010, 449 cases of Marburg virus infections, of which 368 were fatal – an 82% mortality rate [3]. According to the WHO, each year there are approx. 200,000 cases of yellow fever worldwide, of which 30,000 cases are fatal [4], and in the Americas only in 2007, more than 890,000 cases of dengue virus infections were reported [5, 6].

Viral hemorrhagic fevers are most common in Africa, Southeast Asia and South America. In Europe, only CCHFV and hantaviruses occur naturally. However, other diseases can be transferred into Europe by tourists, immigrants or natural vectors [7]. To date, neither specific treatment nor effective prophylaxis against VHFs (excluding yellow fever) has been developed. Usually, only intensive symptomatic and supportive treatment is applied in conditions that ensure the biosecurity of personnel [8]. Lack of specific drugs, possibility of humam-to human transmission and danger of using VHFVs as biological weapons or bioterrorism agents make the search for new strategies for treatment and specific prevention an urgent necessity [9]. As presented below, scientists are making intensive efforts to resolve the problem of VHFs, and exploit the latest discoveries in molecular virology and genetic engineering.

Antiviral substances of natural origin. In recent years, compounds of plant origin have become the object of interest. Among other substances, polysaccharides may be especially attractive as new antivirals. Talarico et al. [10] conducted studies on two sulphated polysaccharides obtained from the Brazilian red seaweeds *Gymnogongrus griffithsie* and *Cryptonemia crenulata* for their antiviral activity against four dengue virus serotypes in different types of cell cultures. These compounds inhibited DENV-2 multiplication in Vero cells (IC_{50} around 1 μg/ml), showed weaker activity against DENV-3 (IC_{50} in the range 13.9-14.2 μg/ml), and DENV-4 (IC_{50} in the range 29.3 to >50 μg/ml), and lack of activity

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against DENV-1. Antiviral effectiveness has manifested in human hepatoma HepG2, foreskin PH, and Vero cells, with the exception of mosquito C6/36 HT cells. The tested compounds were active in the early stages of infection, i.e. adsorption and internalization of the virus, wherein the antiviral effect depended on the cell types and virus serotype. Since it was suspected that heparan sulfate molecules are involved in the binding of the virus particles to the surface of host cells, the scientists focused on compounds that highly resemble anionic heparan sulfate.

Other sulfated compounds of natural origin were involved in research by Ono et al. [11]. Two galactomannans, one extracted from the seeds of *Minosa scabrella* (the ratio of mannose to galactose – 1:1) and the second – obtained from the seeds of *Leucaena leucocephala* (the ratio of mannose to galactose – 1:4) were sulfated (sulfation degree was 0.62 and 0.5, respectively), and tested for their activity against yellow fever and dengue viruses. Studies on infected mice and C6/36 cells showed that the galactomannans can inhibit the adsorption of viruses on the cell surface. Results of the application of mannose-binding lectin chimeric molecule indicate its promising therapeutic potential against Ebola virus [12].

Jain et al. [13] compared the activity of ribavirin, which is a routinely used drug in the treatment of hemorrhagic fevers, with the activity of an extract from leaves of sea buckthorn (*Hippophaë rhamnoides*), used against DENV-2. The extract exerted a protective effect on macrophages, which are the primery target for the virus, and showed immunomodulatory activity.

Reis at al. [14] have tested the antiviral activity of extracts from South American lianas, including *Uncaria tomentosa,* used for medicinal purposes by the Amazonian Indians since ancient times. Its immunomodulatory, anti-inflammatory, cytotoxic and antioxidant properties have been the subject of numerous studies [14, 15, 16]. Inflammatory factors are secreted in large amounts by monocytes in patients with severe dengue fever and increase the permeability of blood vessels, which may contribute to the occurrence of shock. Using flow cytometry, the effect of extracts from the liana on DENV-infected monocytes has been assayed and the reduction of number of infected cells was observed. It was also noted that the extracts had an influence on the release of cytokines and strong immunosuppressive activity.

Liu et al. [17] have received an extract from the Chinese herb *Alternathera philoxcroides*, called the extract No. 1, that after further processing has delivered extract No. 2, identified as oleanolic acid. The following extraction released extract No. 3 containing betaine and extract No. 4, determined as 5-oxoproline. All extracts were tested for activity against 3 strains of Hantaan virus (114, 435, and A9), and it was demonstrated that all of them may inhibit the multiplication of the viruses in Vero-E6 cells. Extract No. 1 was shown to have the highest activity (IC_{50} 153 μg/ml), which is probably the outcome of interaction of all components of the extract. In studies on suckling mice, it was proved that the percentage of survivability of animals dosed with 2,5 mg/ml of the extract on the 3rd, 10th and 14th day after infection was 75%, 50% and 0, respectively. These data indicate that early treatment may effectively inhibit the progress of infection. Since the virus titer in serum and liver was decreased more rapidly than in the kidneys and brain, it was thought that the substances were concentrated primarily in the liver of mice.

The impact of essential oils extracted from *Lippia alba*, *Lippia origanoides*, *Artemisia vulgaris* and *Oreganum vulgare* on yellow fever virus replication became the subject of research conducted by Meneses et al. [18]. Inhibitory activity of the compounds was apparent when the virus was incubated with the essential oils for 24 h before infection of Vero cells, but when the cells were treated with essential oils just before infection, there was no such activity. It is believed that the effectiveness of the essential oils is the result of the direct inactivation of the virus particles, possibly caused by the destruction of the viral envelope by lipophilic compounds. The main components of the essential oils tested in the study were: carvone, carvacrol, limonene and thymol. Clarifying the mechanism of activity of these compounds on viruses should be the aim of further studies.

Synthetic substances. Ribavirin (1-beta-D-ribofuranosyl-1,2,4-thiazole-3-carboxamide) is the only drug routinely used in the treatment of hemorrhagic fevers; however, it is not always effective (it is ineffective in Ebola virus and Marburg virus infections), especially in severe cases, and can also cause serious side-effects. Candurra et al. [19] studied the psychotropic drugs: trifluoperasine (TFP) and chlorpromasine (CPZ) in terms of their impact on replication of arenaviruses: Junin Tacaribe, and Pichinde. It was shown in *in vitro* tests that both compounds in nontoxic concentrations can inhibit the multiplication of the mentioned viruses. While chlorpromasine was affecting the early stages of viral replication, trifluoperasine, beyond blocking virus penetration into cells, also negatively affected the process of virions maturation. Immunofluorescence assay also revealed the influence of both drugs on the distribution of viral particles on the cell surface.

Antiviral activity of N-aryl purines, including N^1-3-fluorophenyl-inosine (FPI) and N^1-3-fluorophenyl-hypoxanthine (FP-Hx) against hantaviruses was tested by Chung et al. [20]. The experiments on Vero-E6 cells showed that FPI was the compound having the higher activity. Since there was no effect on the reduction of the amount of viral RNA, and no increase in the frequency of mutation was noted, it is believed that the activity of the compound may be related to postreplication interaction of FPI with viral or host proteins, affecting the amount of released progeny virions. The search for compounds structurally similar to FPI should be continuated, as the results obtained so far indicate that they may serve as effective inhibitors of virus maturation.

Bray et al. [21] assayed the influence of 3-deazaadenosine (C-c^3Ado) 3-deazaneplanocin A (c^3-Npc A), adenosine analogs on mice infected with Ebola virus. A single dose given shortly after infection prevented the progress of the disease in animals. It was shown that the protective effect was associated with increased production of interferon α in infected mice. The ability of these compounds to block DNA and RNA viruses is probably associated with the inhibition of cellular enzyme S-adenosylhomocysteine hydrolase by diminished methylation of the 5' cap of viral mRNA that causes a decrease of translation of the viral genes. Further studies on the properties of these compounds will allow the determination of its antiviral activity in primates.

It has been shown [22] that many adenosine analogues have the ability to inhibit replication of the Ebola virus, probably by blocking the cellular enzyme S-adenosyl-L-homocysteine hydrolase, which results in an indirectly reduced methylation

of the 5' cap structure of viral mRNA. Administration of one of the adenosine analogues to mice in a dose of 2,2-20 mg/kg for 9 days protected the animals against infection. It was also shown that a single administration of selected analogs in a dose of 80 mg/kg or 1 mg/kg on the first or the second day after infection gave the best therapeutic effect (a 1,000-fold reduction in viral titer). Adenosine analogues were effective in reducing progression of the disease, although they did not completely inhibit the infection.

Recently, a family of small molecules that may selectively inhibit the EBOV and MARV glycoproteins (GP) which mediate the infection of human cells, was discovered [23]. The antiviral activity of the new drugs was tested using a chimeric HIV-Ebola virus, that had an envelope protein of Ebola virus. Compound 8a was identified as a specific inhibitor of the EBOV and MARV entry (IC_{50} 30 μM) into the host cells. The 3-aryl derivatives of this compound showed even greater antiviral activity.

The Crimean Congo hemorrhagic fever virus (CCHFV) is a pathogen that causes disease with a high mortality rate. Simon et al. [24] studied the effect of nitric oxide (NO) on the virus infection. NO plays an important role in the mechanisms of innate immunity in animals. The use of nitric oxide donor – S-nitroso-N-acetylpenicillamine (SNAP) – reduced the number of progeny virions by up to 99%, in comparison with control. Inhibition of the expression of some viral genes and decrease of the concentration of viral RNA in infected cell cultures have been shown. Using the compound SIN-1, a peroxynitrite donor had no significant antiviral effect, which indicates a need for further selection of compounds similar to SNAP.

Barrientos et al. [25] has studied the effect of cyanovirin-N (CV-N) in terms of its antiviral activity against EBOV. After dosing cyanovirin to the infected cell culture it was noted that the development of viral cytopathic effect was inhibited, and the death of infected mice was delayed. The mechanism of activity of CV-N is probably based on its ability to bind to the viral envelope glycoprotein, i.e. $GP_{1,2}$, (to be exact – the ability to bind to mannose-rich oligosaccharides) that intermediates in virus entry into cells. These results may encourage further research on these substances.

The properties of another small-molecule compound, FGI-106, were studied in the *in vitro* model by Aman et al. [26], which were shown to inhibit the multiplication of Ebola virus, Rift Valley fever virus and dengue virus by affecting the replication of the Ebola virus, and other even genetically distant viruses. Therefore, a broad spectrum of activity was observed both in human and mice cells, suggesting that the effect of FGI-106 is associated with the impact on a common pathway utilized by different viruses. Further research may assist in understanding the molecular mechanisms of the antiviral activity of FGI-106, and determine its potential clinical usage.

Studies of Bouloy et al. [27] revealed that pyrazine-carboxamide T-705 is a dose-dependent inhibitor of RVFV. Host cell enzymes (kinases) convert T-705 to T-705RTP (ribofuranosyl phosphate), which inhibits viral RNA-dependent RNA polymerase. *In vitro* experiments showed that this compound was also active against JUNV; additionally, it was less cytotoxic than ribavirin.

Nitrogenated heterocyclic derivatives of imidazothiazoles (compounds having properties of fungicides, herbicides, as well as antitumor activity) were tested in terms of antiviral activity against JUNV. Two of the 12 tested compounds showed activity against JUNV in Vero cells, which was higher than the ribavirin activity [28]. This effect was increased by further structural modifications. The compounds were the most active if they were added to cells simultaneously with the virus.

Smith et al. [29] attempted to determine the significance of heat shock protein 90 (Hsp90) for Ebola virus replication. Chaperone proteins are one of the most important factors influencing the replication of RNA(-) viruses (in polio virus, protein Hsp90 is necessary for proper joining of the capsomeres, and they control the functions of polymerase in paramyxoviruses and bunyaviruses). Efforts have been undertaken to investigate the inhibitors of Hsp90 (both natural and synthetic): geldanamycin, radicicol, 17-allylamino-17-demethoxygeldanamycin (17-AAG; geldanamycin analogue), and a new class of benzamides: AV-1, AV-2, AV- 3 and AV-81, in order to determine their effect on viral replication of EBOV. It was found that each of the tested compounds has the ability to inhibit viral replication, but the benzamide AV 1-3 proved to be the most effective antiviral agent. These properties of Hsp90 inhibitors may result in a breakthrough in the search for new strategies of viral infections treatment.

Sulfated glycosaminoglycans, such as heparin, inhibit the early stages of dengue infection by interactions with viral envelope proteins. Kato et al. [30] found that the chondroitin sulfate E (but not chondroitin sulfate D) effectively inhibited virus adsorption on the surface of BHK-21 and Vero cells, suggesting that the common determinants of these hydrocarbon compounds may be crucial for their inhibitory activity. In the tests, the recombinant envelope protein was binding selectively heparin and chondroitin sulfate E, which proved that the specific structure of the hydrocarbons is more important than the number of negatively charged sulfate groups. Glycosaminoglycans are unbranched sulfated polysaccharides that occur in large numbers on the cell surface. They may have 5 isomeric forms, such as: heparin, heparan sulfate, chondroitin sulfate, dermatan sulfates and keratan sulfate. They are essential for cell growth, maintenance of their integrity and mutual communication. The inhibitory activity of heparin and chondroitin against filoviruses may be related to their degree of sulfation, but the results obtained by Kato et al. [30] may indicate that the hydrocarbon structure of these compounds play a more important role in the mechanism of inhibition.

The activity of acridone derivatives against different strains of Junín virus and dengue virus was tested by Sepúlveda et al. [31]. Naturally occurring and synthetic acridone derivatives have a well known biological activity (including effects against HIV, Epstein-Barr virus and HSV). Among the compounds tested *in vitro*, two N-allyl acridon derivatives (3c and 3f) were active against JUNV (IV4454 strain) and DENV-2 (NGC strain), but the strongest antiviral effect was observed when the compounds were dosed to the Vero cell simultaneously with the virus. These results encourage further research on the antiviral activity of acridone derivatives.

Immunomodulators. Arbidol is a small-molecule immunomodulator discovered and used for the first time in Russia for the treatment and prophylaxis of influenza and other respiratory diseases. The studies on suckling BALB/c mice infected with Haantan virus (HTNV) demonstrated that arbidol increased significantly the percentage of survivals and prolonged their survival time, in comparison with

control. It also affected the reduction of viral titer [32]. The mechanism of its activity relies on stimulation of interferon secretion and inhibition of fusion of the enveloped viruses with the cell membrane.

In vitro studies on Vero and MRC-5 cells showed that arbidol blocks early stages of multiplication of Chikungunya (CHIKV) [33]. In order to determine more precisely the mechanism of activity of the compound, ARB-resistant mutants of the virus were used. Sequencing revealed a single amino-acid substitution in the region of virus envelope protein, which may be important for the interaction of alphaviruses with cellular receptors. To confirm the role of this mutation on the molecular mechanism of resistance to arbidol, a clone having the same mutation was designed. *In vitro* studies have shown that arbidol impaired the early phase of virus infection (adsorption or penetration). Administration of the compound before infection reduced significantly the viral titer, while dosing it after infection did not affect the titer of the virus. Arbidol has a broad spectrum of antiviral activity against both enveloped and non-enveloped viruses. *In vitro* studies on HCV model showed that the arbidol is associated with inhibition of conformational changes in glycoprotein which are necessary for membrane fusion. In the case of influenza virus, it inhibited viral entry into the cells through destabilization of hemagglutinin, and by blocking fusion with the host's membrane.

Vaccines. Because of the antigenic diversity of etiological agents of hemorrhagic fevers, it is difficult to obtain a universal and effective vaccine. Therefore, scientists are focusing on vaccines that might give protect against particular viral serotypes/genotypes. To receive a vaccine against serotype 1 of denque virus (DENV-1), Maves et al. [34] used viruses inactivated with psoralens, i.e. photoactive compounds that easily penetrate the lipid envelope of the virus, intercalate between pyrimidines and combine them under exposure to UVA light, making it possible to inactivate the virus without affecting its epitopes. A Westpac 74 strain of DENV-1, prepared by the described method, was used to vaccinate monkeys *Aotus nancymaae*. In all immunized animals, the presence of antibodies against DENV was noted, and although viremia due to infection was found in 3 out of 7 immunized monkeys, its duration was shorter than usual (from 3.67 days to 0.71 days).

Rift Valley fever is characterized by a high mortality rate in fetuses and newborns of cattle, goats, sheep, camels and humans. It is one of the most common zoonoses in Africa. For the purposes of prevention, scientists undertook projects on vaccines attenuated or inactivated by formalin. For example, inactivated Smithburn strain proved to be highly immunogenic, but in cattle and sheep it showed teratogenicity during pregnancy. Other vaccines used which gave promising results in animals were prepared from Clone 13, an attenuated mild strain obtained from human cases in Africa, and from MP12, which is an attenuated Egyptian isolate [35]. MP12 is now tested in the second phase of clinical trials. Vaccines containing formalin inactivated RVFV are not registered and are not commercially available. They are available only for vets working in endemic areas, laboratory workers, and other high-risk groups. Inactivated vaccines are expensive, difficult to prepare, and require both a higher dose in comparison with attenuated vaccines, and annual boosters to maintain an adequate level of immunity [36].

Rao et al. [37] investigated the possibility of inducing cytotoxic T lymphocytes (CTLs) in mice by infection with irradiated Ebola virus, or with the same virus enclosed in liposomes that contain lipid A. It was shown that intravenous immunization of mice can induce CTLs, but the effect of using irradiated virus was not stable, in contrast to the viruses enfolded in liposomes with lipid A. It is suggested that liposomal lipid A may have the activity of an adjuvant. This discovery and recognition of specific epitopes at the C-end of viral GP are promising steps in the development of vaccines against Ebola virus.

Another strategy to develop vaccines against hemorrhagic fevers viruses may be the use of virus-like particles (VLPs), which mimic the viral structural proteins, but do not contain the viral genetic material. VLPs can be produced in different types of cell cultures, both in mammalian and insect cell lines, yeast and plants. These particles have a morphology similar to wild-type virus. They present viral antigens in native conformation, and are therefore easily recognized by the immune system [38, 39].

Sun et al. [40] tested the potential of recombinant VLPs produced in insect cells with the use of baculoviruses expression system. Their study showed that double immunization of mice with 50 μg of Ebola-VLPs induced the production of high levels of antibodies for Ebola GP, providing complete protection. It was also found that the addition of adjuvant allowed a significant reduction in the dose of Ebola-VLPs, while providing the same level of protection. Näslund et al. [35] utilized VLPs produced in mammalian cells for triple immunization of mice, yielding high titers of antibodies that protected the animals against infection with a lethal dose of Rift Valley fever virus. Vaccination with VLPs inhibited efficiently RVFV replication without side-effects. The value of chimeric virus-like particles containing RVFV glycoproteins G_N and G_C and gag protein of Moloney murine leukemia virus (MoMuLV) was studied by Mandell et al. [36], who assessed the effectiveness of vaccines based on the RVFV-VLPs, using 2 animal models. Immunized mice were partially protected against infection, while the vaccinated rats were completely resistant to a lethal dose of RVFV. This indicates that the strategy of using VLPs may be an important step in searching for new ways to combat HFVs infections [40].

Efforts are also being made to use recombinant vaccines against Marburg virus (MARV). A recombinant vaccine was prepared on the basis of vesicular stomatitis virus (VSV) containing an insertion of gene encoding glycoprotein of Musoke strain of MARV. Daddario-DiCaprio et al. [41] examined the effectiveness of this vaccine in tests on monkeys infected with 2 strains of MARV, i.e. Musoke and Ravn. The immunized and infected animals showed no symptoms of disease, which confirmed the suitability of this type of vaccine to protect from different strains of Marburg virus.

Feldman et al. [42] studied the efficacy of another recombinant vaccine based on VSV vector that expressed the Ebola virus glycoprotein. Studies conducted on guinea pigs, mice and monkeys have shown that the survival rate of guinea pigs immunized with the vaccine 24 h after infection was 50%, and in mice even reached 100%. When the vaccine was dosed to monkeys 20-30 min after infection, 50% of them turned out to be protected. Further research may deliver a promising strategy in the fight against infections with filoviruses.

Geisbert et al. [43] studied the efficacy of a recombinant vaccine based on VSV and encoded glycoprotein (GP) of Marburg virus. After its administration to monkeys, 24 h after infection with a lethal dose of the virus, 5 of 6 animals survived. When the vaccine was given 48 h after infection, 1/3 of the animals turned out to be sufficiently protected. These results can be used in further research on the prevention against Marburg virus infections in humans.

It has been shown [44] that a vaccine designed against 2 strains of Ebola (EBOV Zaire and EBOV Sudan) protected the monkeys not only against those strains, but it was also effective against the newly identified strain of the virus, i.e. Bundibugyo (BEBOV). The immunization strategy called 'prime-boost' relies on the fact that the animals were vaccinated for the first time with DNA encoding a viral glycoprotein of EBOV (Zaire and Sudan strains), and after a year they were immunized again with a vaccine based on recombinant adenovirus encoding surface glycoprotein of ZEBOV. After the second immunization, the animals were infected with a lethal dose of the virus and none of them showed symptoms of the disease. In immunized animals, strong cellular and humoral response against GP of ZEBOV and GP of BEBOV have been reported, even though the vaccine did not contain any antigens of BEBOV in its composition.

The 17D vaccine containing live attenuated yellow fever virus is considered one of the most effective and safe vaccines. It has been used for over 70 years in the prevention of the disease, and a single dose was shown to cause a long-term resistance (approx. 10 years). However, there have been occurrences of side-effects, especially in vulnerable groups, such as adults over 60 years of age, children up to 6 months, people with decreased immunity or with HIV, which constitutes a barrier against protection from yellow fever in its endemic areas where HIV infection is very common. Furthermore, reports have shown cases of hypersensitivity reaction in people sensitive to components of the vaccine, neurotrophic disease (YEL-AND), and YEL-AVD, which motivates scientists to search for new solutions of effective and safe prevention [42]. Monath et al. [46] studied the effect of an inactivated vaccine consisting of 17D yellow fever virus that had been inactivated with β-propiolactone. The vaccine was tested on 60 healthy people aged between 18-49 years. One group received the vaccine at a dose of 0.48 μg, the second received 4.8 μg, and the immunization was repeated after 21 days. The results showed that the vaccine caused the appearance of antibodies in 100% of the people who received the higher dose of antigen in each injection, and in 88% of the people who were immunized with the dose of 0.48 mg of antigen in each injection. In both groups, 3 types of side-effects were noted, i.e., mild pain, tenderness and itching at the injection site, and one case of urticaria was reported on the third day after the second 4.8 μg dose. The results indicate that the vaccine containing the inactivated antigen with alum adjuvant may be a safer alternative to live attenuated 17D vaccine.

Intensive research is being conducted to obtain vaccines against individual genotypes of hantaviruses. In South Korea, the living atenuated vaccine against Hantaan serotype ('Hantavax') is routinely used, even in the Korean army, with the effectiveness confirmed in controlled clinical studies [47]. Moreover, there are effective vaccines against Argentine and Bolivian VHFVs. In Bulgaria, in risk groups, vaccine against CCHF is used.

Other possibilities. In the search for effective prevention of hemorrhagic fevers, the small interfering RNA (siRNA) technique is also under intensive study since it enables inhibition of gene expression and replication of the virus [48]. When choosing this type of therapy, the main difficulties are related to the method of delivery of siRNA into target cells *in vivo*. One possible solution might be the use of SNALPs, i.e. a specific siRNA attached to the liposomes, forming acid-lipid particles. To test whether siRNA inhibits replication of ZEBOV, *in vitro* Vero cells were transfected with a pool of 4 different siRNAs designed for specific silencing the L gene of ZEBOV. Transfected cells were infected at 0 h, 24 h and 48 h; 24 h after infection the medium was collected to determine the viral titer. Depending on the time of siRNA transfection and infections, the production of ZEBOV progeny virions was inhibited from 2-10 times. Experiments in guinea pigs have shown that siRNAs delivered with poliethylenimine shortly before infection caused the decrease of viral titer, and partially protected the animals from death. The use of siRNAs delivered as SNALPs gave the strongest antiviral effect, that was manifested by decrease of viral titer and protection of all animals from death. The prospect of application of siRNAs in the treatment of viral infections arouses great hope today [48], especially in terms of the possibility of using cocktails of siRNAs in combination with other products, such as immunoglobulins. In subsequent studies, Geisbert et al. [1] assayed the siRNAs targeted to the polymerase L and 2 viral proteins (VP24 and VP35) of ZEBOV to determine their antiviral activity. In the form of SNALPs, the siRNA pool was tested on monkeys (*Rhesus macaques*) which received the mixture at a dose of 2 mg/kg 30 min after infection, and thereafter on the 1st, 3rd and 5th day. The result was a 66% survival in this group of specimens. In the second group, which received 7 doses of the siRNA pool 30 min after infection, and daily for 6 subsequent days, a 100% survival of animals was observed. The outcome proved that siRNA may be a useful tool in antiviral treatment strategy after exposure to virus.

Fowler et al. [49] utilized the phenomenon of silencing interference of RNA with the expression of genes encoding 3 structural proteins: NP, VP35 and VP30, that take part in the formation of nucleocapsid of Marburg virus (MARV). Experiments were conducted *in vitro* on HeLa and Vero cells. Small interfering RNAs homologous to the 3 transcripts (NP, VP30, VP35) were administered into the cells with a plasmid encoding the mentioned nucleocapsid proteins, after which the level of synthesis of particular proteins was determined. As a result, a significant reduction in the levels of all 3 proteins was observed, and a decrease in the amount of released progeny virions was noted.

Enterlein et al. [50] investigated the antiviral activity of phosphorodiamidate morpholino oligomers (PMOs) against the Ebola virus. These compounds are analogues of single-stranded DNA modified in such a way that each subunit contains a morpholine ring phosphorodiamidate linkage instead of phosphodiester internucleoside. They combine in a complementary manner with mRNA translation initiation sites, and block the translation process. To increase the chance of internalization into cells, the PMOs were conjugated with arginine-rich peptides (P-PMOs). The PMOs and P-PMOs used in the studies were designed for 6 sequences of ZEBOV sense or antisense RNA. PMOs combined with peptides, as well as in a free form, were screened in terms of antiviral

activity *in vitro*, and after selection of the most effective compounds, they were subjected to assays on mice. It was found that P-PMO specific for VP35 sequence inhibited the replication of the virus in cell culture and increased the survivability of infected animals, both when was administered prophylactically and therapeutically. PMO unconjugated with peptides also showed prophylactic activity in mice, which suggests that these particles may be a new strategy in the prevention and treatment of EBOV infections. Warren at al. [51] also showed that positively-charged PMO (PMOplus) demonstrated postexposure protection of monkeys infected with Ebola and Marburg viruses. This is a promising example of the application of advanced antisense therapy in postexposure prophylaxis in *Filoviridae* infections.

Takada et al. [52] evaluated the effectiveness of 2 neutralizing monoclonal antibodies specific for GP of ZEBOV. Their epitopes are located in a relatively conservative region of GP. Mice that received the antibody on the second day after infection were immune to infection, whereas animals treated on days 3rd and 4th were only partially protected. Early treatment with humanized monoclonal antibodies can be an effective strategy against Ebola virus.

In combating the symptoms of West Nile virus infection, it was shown that humanized rodent antibodies Hu-E16, directed to the highly conserved domain III of the envelope protein, have high antiviral efficacy. Although the antibody therapy is promising, the possibility of its wide use is limited by high production cost. In order to reduce this, a cheap method of obtaining the antibodies in genetically modified tobacco *Nicotiana benthamiana* was developed [53], and in tests it was proved that the antibodies from plants had activity identical to those ones obtained from rodents.

Viral infection begins with penetration of viral particles to the cells, whereas viral envelope glycoprotein (GP) plays a crucial role in the fusion of virus with the host cell membrane. Miller et al. [54] studied the peptides which effectively inhibited Ebola virus entry into cells through an interaction with viral GP. The greatest activity was observed when Vero cells were treated with peptides 30-60 min prior to infection.

In the treatment of infections caused by Ebola virus, an innovative attitude that relies on interference with the disease process rather than replication of the virus, may be a useful approach [55]. Due to the fact that infection with Ebola in primates induces hyperexpression of procoagulant in monocytes and macrophages, it was assumed that the inhibition of coagulation factor pathway may alleviate the symptoms of EBOV infection. The recombinant anticoagulant protein c2 (rNAPc2) of nematodes, which is a strong inhibitor of tissue factor crucial to blood coagulation, was administrated to the infected animals. In experiments on monkeys, c2 was given 10 min after infection (first group), or 24 h after infection (second group), and it was observed that the duration of survival in both groups was extended by up to 33%.

Carette et al. [56] have identified a cellular protein that plays a key role in EBOV infection: Niemann-Pick C1 protein (NPC1) This protein is present in the membrane of lysosomes, participates in the transport of cholesterol, and mediates the 'escape' of viral particles from lysosomes to the cytoplasm. Lack of this protein due to mutations, causes a rare degenerative illness called Niemann-Pick disease. Most of the mice with mutation in NPC1 survived infection with a lethal dose of Ebola virus, similarly to fibroblasts derived from patients suffering from this disease. Studies using other viral models showed that only EBOV and MARV require NPC1 for infection. This discovery gives new possibilities in the treatment of these hemorrhagic fevers.

SUMMARY

Finding effective means for combating and preventing hemorrhagic fevers is now an urgent necessity. Lack of active drugs against viruses that cause these diseases, high risk of death in patients, animals' illnesses causing economic losses, as well as the possibility of transmitting infection outside the endemic areas, strongly encourage scientist to search for new vaccines and antiviral preparations, making this type of research well-grounded. The presently reviewed investigations, which focus on natural and synthetic substances [57] or genetically engineered vaccines, are promising and offer hope to overcome these diseases. Experiments performed both *in vitro* and *in vivo* proved to be successful in many cases, thereby encouraging further research, including clinical trials. Lack of active drugs makes the search for substances allowing the extension of life of infected animals of the utmost importance, because they may extend the time to apply various therapeutic methods and increase chances of complete recovery.

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