Open Source Center Analysis

Recent Worldwide Research on Animal Pox Viruses

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Executive Summary

Although smallpox disease has been eliminated worldwide, concerns remain regarding variola virus (the cause of smallpox) and related poxviruses. It is widely feared that samples of variola virus may have been retained in several countries after a WHO directive to allow storage of such samples only in Russia and the United States. Even if the US and Russian stocks of variola virus were now destroyed, there would still be concern over possible hidden collections. A further worry is that some of the stocks of variola virus may have found their way into the hands of terrorists or rogue nations. It is even possible that the virus could be obtained from sites of human burials and grown up in a laboratory. These concerns are compounded by the suspension of vaccination against smallpox over the past three decades – with the eradication of smallpox as a naturally occurring disease – resulting in a population that lacks adequate immunity to the deadly virus.

With advances in molecular genetics technologies as well as in understanding of the disease-causing mechanisms of poxviruses, it is now possible to genetically engineer relatively benign poxviruses to make them more virulent. As a result, several available poxviruses already able to infect humans (monkeypox, buffalopox, cowpox, camelpox, and the vaccinia-like viruses circulating in the wild in Brazil) could potentially become serious human pathogens through evolutionary changes or laboratory manipulations, and perhaps become as worrisome as historical smallpox disease. Current vaccines will probably protect against the naturally occurring viruses as they evolve, but they might not protect against certain genetically engineered viruses.

The possibility that a poxvirus could be made more virulent for humans increased in likelihood when scientists in Australia found that a soluble factor of the immune system (called IL4) markedly worsened a poxvirus disease in mice. In that case, incorporation of the mouse IL4 gene into the mousepox virus genome led to fatal disease even in mice that normally had a genetic resistance to mousepox. Of great concern, the mousepox virus carrying the IL4 gene induced fatal disease in mice despite prior standard immunization against mousepox virus (immunization that protected mice from an unmanipulated mousepox virus). Therefore, it is possible that genetic modification of a poxvirus that causes mild human disease (vaccinia, monkeypox, cowpox, camelpox) might produce an engineered poxvirus that is as lethal as variola virus. Moreover, it is feared that such an engineered virus might cause fatal disease even in immunized people.

Apart from the Australian experiment, other countries have bioengineered Orthopoxviruses (vaccinia, monkeypox, cowpox, camelpox) by introducing different gene coding for different cytokines. In France, for example, scientists introduced IL2 genes into a vaccinia virus in order to study treatment of cancers. In Spain, researchers bioengineered the same virus with genes that produce two cytokines: IL8 and IL12. Japanese scientists did the same experiment using genes encoding for IL12, IL17 and IL23. The Chinese, on the other hand, separately engineered a human interleukin gene

and an interferon gene into an Othopoxvirus. Such experimentation could potentially lead to the creation of a super poxvirus with enhanced pathogenecity and increased lethality. In the wrong hands such an "enhanced" virus could readily be used as a BW agent.

The intensive Orthovirus genetics program that existed under the Soviet regime gives rise to concerns that advanced technology could transfer from Russia to countries harboring anti-US sentiments, third-world countries, terrorist organizations and/or non-state actors. Of utmost concern are Russian scientists with experience in Orthovirus bioengineering who have moved to other countries and continue to pursue this research topic. For example, an Australian paper on substituting vaccinia virus for variola virus for Orthoviruses research had six Russian scientists' names on it. Although the point of the experiment was to detect vaccinia virions in aerosolized material, this research shows knowledge of substituting vaccinia for variola and aerosolizing droplets (i.e. proliferation), then sampling and detecting for long-term monitoring of personnel exposure in a bioaerosol-contaminated environment (i.e. couunterproliferation). Under the right circumstances similar scientists could be persuaded to work for rogue entities or governments, thus raising the level of concern that poxviruses could be used in a bioweapon.

As a result of generalized concern about smallpox or a related virus as a bioterrorism agent, many countries have established detection assays and public health disaster plans to counter such an event. Some of these countries have begun to stockpile vaccines and to develop anti-viral drugs that might be effective should a poxvirus event occur.

Many Americans were vaccinated against smallpox decades ago, and it is likely that of these some individuals are no longer protected, some are partially protected and would therefore get mild disease if infected, and some are fully protected. The great majority of Americans born after 1972 were not immunized and would not be protected. The old vaccine, however, is too toxic in too many people to be given to everyone in a non-crisis situation. A newer vaccine with far fewer side effects might be acceptable if needed.

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Analysis

Naturally occurring smallpox disease was eliminated worldwide in 1977. Routine vaccination of US civilians against smallpox was discontinued in 1971, but allowed for travelers to endemic regions until the late 1970s. In most other countries, vaccination of the general population ended by 1982. As a result of this halt in vaccination, most of the US population could now become ill with smallpox disease should it be reintroduced by accident or intentionally. In addition, humans are susceptible to several naturally occurring viruses related to smallpox, one of which could become a serious disease risk through natural evolution. Routine smallpox vaccination previously protected against these viruses. Finally, there is concern about the potential creation of a genetically engineered poxvirus that might be markedly pathogenic for humans.

When smallpox was being eradicated worldwide, the World Health Organization (WHO) moved to eliminate the causative variola virus from all laboratories and strain collections. The first step in 1984 was to confine variola virus samples to two centers, one in the United States and one in Russia. However, whether all other strain collections were destroyed or turned over to those two centers has been a matter of controversy and concern. It was feared that certain nations may have decided to retain samples of variola virus. At least as likely was the possibility that viral collections that included samples of variola viruses may have been retained by individual scientists in various countries through (i) defiance, (ii) laziness in not searching for variola samples, or (iii) inertia, despite knowing that such samples were present. The next step for the WHO was to propose eliminating variola virus samples even from the two designated centers. The United States balked, concerned that Russia might not destroy all of its stocks and that other collections of variola virus might still exist clandestinely. The United States argued that additional research was needed to better understand the virus in order to develop suitable anti-smallpox drugs that might be used should the disease recur for whatever reason. Indeed, laboratory security for serious pathogens throughout much of the world, including the United States, was rather lax until the recent past. As a result, thefts of worrisome bioagents could have been carried out many times, an issue with regard to potential reintroduction of smallpox disease into the human population from pilfered viruses (1).

A defector from Russia, who had been involved in biological warfare (BW) activities, stated and published that Russia had had a secret BW research program that included variola virus work and that the program had culminated in the weaponization of the virus for BW delivery (2). This testimony, along with the hint by Alibek that hidden stocks of smallpox virus might exist, increased the resolve of US officials to retain its samples of variola virus for defensive research. That position was supported by a study of the US National Academy of Sciences, which recommended areas of research that should be done with live variola virus, considering the bio-threat potential (3).

A Russian paper published in 2006 (4) stated that “smallpox virus is kept in laboratories of *some* or *certain* countries.” [Italics added for emphasis.] This wording hints at possible Russian knowledge of variola virus stocks in countries other than the United

States and Russia, but may merely refer to the two countries known to have such stocks: Russia and the United States. Another Russian paper states that according to American experts: "at least 10 countries have such collections and this, through accidental leaks [spillages], could possibly endanger the entire world health protection system" (5). Indeed, many other countries are concerned about the existence of unofficial caches of variola virus (6-14).

Russian scientists at VECTOR created plasmids each carrying the complete genomes of one of thirteen strains of variola virus (15-17). Such plasmids could potentially be employed to reconstitute a viable variola virus or used to create a modified virus with unusual properties. Moreover, those plasmids could be used to separately transport non-infectious parts of variola virus for later reassembly. It is recognized that such a process would require sophisticated molecular virology procedures.

Concerns about *Orthopoxviruses* (the group of viruses that comprises variola virus and the poxviruses most closely related to variola virus) include the following:

1. *Orthopoxviruses* are stable and can be stored for long periods of time. A small sample of an *Orthopoxvirus* can be grown up in a suitable laboratory so that large batches of viruses are obtained.
2. Hidden caches of variola viruses that should have been destroyed decades ago, but which may not have been destroyed, might exist and be tapped to grow up large amounts of virus for potential use in a terrorist event (8).
3. Stocks of variola virus held in Russia might have been raided at a time in the past when security was lax, or taken by Russian laboratory workers leaving for jobs in other countries (7). A portion of such viral stocks might be grown up and used by terrorist groups.
4. Current Russian variola stocks might be diverted to terrorist groups or rogue states.
5. A virulent monkeypox virus (already a pathogen for humans) might be altered by natural means or by genetic engineering in a laboratory to make it more virulent, perhaps even as virulent as variola (smallpox) virus (18). For example, the gene encoding the IL1*beta*-related protein present in monkeypox virus but absent in variola virus could be deleted in monkeypox virus, which might make the resulting virus more deadly (19).
6. Another virus related to variola virus, such as cowpox virus, camelpox virus, or any of several vaccinia viruses, might be modified genetically so as to make it a serious pathogen. These genetic modifications could be mediated through *in vitro* recombination experiments, by random mutagenesis, or by directed addition of virulence genes.
7. A number of *Orthopoxviruses* are able to infect humans (e.g., cowpox, monkeypox, vaccinia variants, camelpox). Aside from variola and certain strains of monkeypox virus, they tend not to be passed efficiently from human to human by droplet transmission and they cause relatively mild disease. However, various genetic manipulations, including (but not limited to) the addition of a cytokine gene, such as that encoding interleukin 4 (IL4), might turn an *Orthopoxvirus* that

causes only mild to moderate disease in humans into a lethal infection. It is already known that recombinant vaccinia virus carrying the mouse IL4 gene is lethal for mice (20).

When smallpox was a widespread disease, a certain number of adverse effects of the vaccine were tolerated. However, a need for new, safer vaccines has arisen because the adverse events from the old vaccines were deemed unacceptable in the absence of the disease.

Worldwide concerns have caused many countries to disseminate response plans in case of an *Orthopoxvirus* outbreak, develop new vaccines (21-29), and construct mathematical models of disease spread (14, 30-40).

Immunological Aspects of Poxvirus Infections

The mammalian immune system uses many soluble mediators to protect against a variety of infections. One of these soluble mediators, IL4, is produced by a subset of T lymphocytes (called Th2 cells) during an immune response. IL4 promotes antibody production but interferes with the development of T cell mediated immunity, including the formation of cytotoxic T cells and production of interferon-gamma. However, cytotoxic T cells and interferon-gamma are critical to eliminating viruses from the body during a viral infection. Consistent with these concepts, IL4 expression caused a delay in vaccinia virus clearance from target organs (41). In studies with far-reaching implications, Australian scientists engineered the gene encoding mouse IL4 into mousepox virus (also called ectromelia virus) and discovered that the mousepox virus bearing the IL4 gene had very different properties than ordinary mousepox virus (41, 42). **The IL4-bearing mousepox virus acted as a super-pathogen**. It caused disease in mouse strains normally genetically resistant to mousepox, leading to a high mortality. This finding with the IL4-modified mousepox virus has spread a fear that a genetically engineered *Orthopoxvirus* able to infect humans, such as vaccinia virus or cowpox virus, might be made more virulent for humans by similar genetic engineering. IL4 carrying vaccinia virus was found to be lethal for mice (20). It should be noted that human IL4 and mouse IL4 have structural differences (43). As a result, the effects on mice of a vaccinia virus carrying the mouse IL 4 gene may be species specific (43, 44). However, it is likely that a vaccinia virus carrying the gene for human IL4 would be a super pathogen in humans.

In the Australian studies, the mouse IL4 gene was incorporated into the mousepox virus, and the genetically modified virus administered to laboratory mice. That single genetic alteration (addition of the gene encoding IL4) led to fatal disease in all strains of mice tested. Of perhaps even greater concern, the IL4-bearing mousepox virus overcame standard immunizations that normally protect mice from mousepox disease. That is, mice vaccinated against mousepox resisted infection with the usual mousepox virus but contracted the fatal disease when they were given a mousepox virus genetically engineered to carry the mouse IL4 gene (42). The failure of immunization to protect

mice against such an engineered poxvirus suggests that the IL4-bearing virus interfered with the expression of memory immune responses. Indeed, the physiological basis of the IL4 effect is largely understood. It appears that two kinds of cells of the immune system, NK cells and cytotoxic CD8+ T cells, mediate resistance to mousepox virus both by direct cellular action and through production of interferon-gamma (42, 45). Apparently, IL4 inhibited these two cell types critical for clearing virus-infected host cells. The cytotoxic CD8+ T cells were indirectly inhibited by IL4. IL4 suppressed the development of Th1 type T cells (41). Such suppression interfered with generation of CD8+ cytotoxic T cells. Overall, the marked over-expression of IL4 caused by the genetically engineered viruses prevented viral clearance and markedly worsened the disease. It was also noted that the antiviral drug cidofovir failed to protect mice from the mousepox virus that bore the mouse IL4 gene (46).

This set of results indicates that it is entirely possible that genetic modification of an *Orthopoxvirus* that causes mild human disease (vaccinia, monkeypox, cowpox, camelpox, etc.) might produce an *Orthopoxvirus* that causes a disease in humans with the severity of smallpox. Thus, one might not even need to obtain samples of variola virus to create a serious poxvirus outbreak – modification of a readily available poxvirus might serve such a purpose. Moreover, an engineered *Orthopoxvirus* might even be virulent in people immunized against smallpox and other poxviruses. Therefore, experiments in which immune mediators are engineered into *Orthopoxviruses* should be viewed with concern.

As part of a substantial research program involving poxviruses, French scientists engineered human IL2 into vaccinia virus to study feline fibrosarcomas (47). Although that work is directed at treatment of cancers (47), such experiments are worrisome. They would be of greater concern if an adversary had performed them, because interleukins have the potential to make *Orthopoxviruses*, (including vaccinia viruses, monkeypox virus, cowpox virus, camelpox virus and even variola virus itself) more virulent than they otherwise would be. In other experiments, Spanish scientists engineered vaccinia virus to carry the genes encoding IL12 and IL18. Their studies demonstrated that those two cytokines facilitate the clearance of the virus (48). However, it is of concern that cytokines are being engineered into vaccinia viruses and perhaps other *Orthopoxviruses*.

Several *Orthopoxviruses* were found to carry genes encoding proteins able to bind IL18 (49, 50). The binding of IL18 increased virulence (50). Other methods of inhibition of IL18 also were found to increase virulence (56), which could point to various approaches to interfering with IL18 so as to render a poxvirus infection far more serious. Other *Orthopoxvirus* virulence factors also have been studied (52-58). Manipulation of some of these could possibly increase virulence.

Japanese scientists genetically engineered vaccinia viruses to bear human cytokine genes (59). The cytokine genes included those encoding IL12, IL23, and IL17 (59). Although none of these constructs are of direct concern, engineering of interleukin genes into a poxvirus pathogenic for humans is worrisome. These studies further found that IL17 and IL23 are important in resisting the virulence of the virus (59). Therefore, interfering with

IL17 and/or IL23 host responses could increase the severity of an *Orthopoxvirus* infection of humans.

Although general fears about potential genetically engineered viral and bacterial pathogens may be excessive in many circumstances, the cautions about an engineered *Orthopoxvirus* carrying a cytokine gene cannot be overemphasized. That is because one of the readily available relatives of the variola (smallpox) virus could possibly be made into a virus lethal to humans by such a maneuver.

A variety of host genetic factors – including but not limited to a preprogrammed suite of cytokine responses – may play a role in the relative susceptibility of different people to specific *Orthopoxviruses*. For example, a certain major histocompatibility complex type (HLA-A*201) has been associated with cytotoxic T cell responses to a variola virus epitope (24). This trait may be one factor, along with preprogrammed cytokine production profiles and other factors, that underlies differences among people or groups of people in susceptibility to the virus.

In addition to its role as a vaccine for smallpox, vaccinia virus has been used as a vector for genetic engineering to deliver genes and/or to function as a carrier in immunizations against a variety of diseases, including HIV and many other infections (60-88). Vaccinia is especially useful in this regard because it can be genetically engineered without loss of its ability to replicate, and the virus can take a large amount of additional, engineered DNA. This function represents a potential dual use for vaccinia viruses.

During Soviet times, Russian scientists intensively studied *Orthopoxviruses*. They did report the first complete sequencing of a virulent variola strain in 1992 (89); however, the depth and extent of the Russian work may not be reflected in the open literature. Although the United States sequenced a different variola strain a year after the Russians, it is only in relatively recent years that other countries, including the United States, have carried out systematic and comparative genetic analyses of *Orthopoxviruses*. As a result of its long commitment to understanding the genetics of variola and related viruses, Russia has substantial experience to draw upon in terms of manipulating *Orthopoxviruses*. That represents an important basis of concern for the United States in terms of (*i*) agreeing to the destruction of all variola virus stocks, and (*ii*) the possibility of creation of genetically manipulated *Orthopoxviruses* by an adversary. The second item includes but is not limited to the possibility of using genetic engineering to add to a naturally occurring *Orthopoxvirus* pathogenic for humans (such as a relatively virulent strain of monkeypox virus or cowpox virus or a now naturally occurring vaccinia virus variant) an immune-modulating gene or genes (such as the gene encoding human IL4) so as to construct a virus more virulent for humans. It should be noted that *Orthopoxviruses* are very large and able to readily accept large amounts of additional DNA by genetic engineering without a need for the very sophisticated methods sometimes required in the manipulation of smaller viruses with RNA genomes.

Camelpox virus rarely induces illness in people, and then typically only a mild case occurs, although the virus causes a serious and often lethal disease in camels. The full

genetic sequencing and analysis of camelpox virus led to the discovery that the camelpox virus is most closely related to variola virus, the cause of smallpox (90). Indeed, variola virus and camelpox virus produce the same kind of small white pocks on the chorioallantoic membranes of fertilized hens' eggs and have a similar ceiling temperature for the growth of such pocks. The two viruses are unable to grow in rabbit skin. Genetic comparisons indicate that (i) the genome sequence of the two viruses are closer than either is to any other virus, (ii) the arrangements of the open reading frames (protein coding sequences) are most similar to each other, (iii) the protein sequences themselves are most closely related to each other, and (iv) the nature and structure of the terminal repeats are most similar between variola and camelpox viruses (90). As camelpox is endemic in regions with large numbers of camels, the discovery of this close-relatedness between variola virus and camelpox virus triggered an immediate concern that terrorists or rogue states might first acquire camelpox virus from natural sources in their territories and then, somehow, convert the camelpox virus into a virus lethal for humans. Although this concern is appropriate, and it might be possible to convert camelpox virus into a variola-like virus lethal to humans, it also might take a substantial research program to turn camelpox virus into a new human pathogen. How many differences between camelpox virus and variola virus are responsible for the current modest virulence of camelpox virus for humans remains unclear. Stated another way, the number of genetic alterations that would have to be made in camelpox virus to convert it into a worrisome human pathogen is uncertain. It might be necessary to make modifications in many of the individual host-range genes of camelpox virus to convert that virus into a serious human pathogen. However, it also may be possible to add one or a few cytokine or cytokine receptor genes to achieve that goal.

In a study conducted by British and Irish scientists, it was observed that camelpox virus encodes a Schlafen-like protein that has mixed effects on virulence and is a gene that is disrupted in variola virus (91). It is conceivable that this gene inhibits virulence and that its disruption in camelpox virus might possibly make the latter more virulent for humans.

Over the years, Russian scientists sequenced the DNA genomes of many *Orthopoxviruses* and compared the encoded protein sequences (89, 92). For example, a 1995 paper compared in detail the sequences of a variola virus strain that had caused an outbreak of smallpox in India in 1967 with a variola strain that had caused an outbreak in Bangladesh in 1975 (93). Alignment of the DNA genomes showed a 99.3% homology. Of 200 protein sequences, 122 were identical, 42 had a substitution in a single amino acid, 11 had changes in two amino acids, and the other 25 had more extensive differences. It was noted that a region of conservation in variola virus is variable in vaccinia viruses (93). Such studies can assist in forensic analyses and in construction of virus variants.

The years of Russian work on poxviruses provided them with insights into the mechanisms by which *Orthopoxviruses* elude host immune responses. Such insights could have given Russian scientists clues to genetic alterations of *Orthopoxviruses* that might make them more virulent. This idea has been magnified by the Australian report of a marked increase in disease severity and mortality in mice after infection with a mousepox virus engineered to carry the gene for mouse IL4 (42).

As time passes, however, and modern molecular genetics techniques become both more sophisticated and disseminated throughout the world, whatever advantages the Russians may once have had should gradually have been reduced or even lost. This is attributed, in part, to a marked reduction in funding for biological research in Russia during the 1990s. That loss of research money led to Russia's failure to develop a modern biotechnology industry and the desertion of many biological scientists. It also impaired Russia's ability to build on its viral research. Indeed, recent studies of *Orthopoxviruses* from the United Kingdom, Australia, and France appear to be as sophisticated as the recent Russian studies or more so. Even the papers from Brazil are as advanced as those of the Russians.

Scientific collaborations between US and Russian scientists are also to be considered. Some such collaborations have been supported by US funds, and were designed in part to usefully employ Russian scientists formerly involved in Soviet-Russian BW programs. These relatively recent US-Russian collaborative studies have included genetic analyses of variola virus strains and related efforts (89, 94). In addition to that work, Russian scientists have independently published, in recent years, results of sequencing of a number of *Orthopoxviruses* (92, 95-97). These studies have provided insights into virulence mechanisms and *Orthopoxvirus* evolution (95, 97, 98). It is unclear how much of this work was performed recently and how much was carried out many years ago and was not published at the time, but has now been updated and published as new work. It is also possible that in addition to providing funds for useful employment of Russian scientists previously working in areas of concern, US scientific interactions with the Russian scientists could possibly have contributed to a greater sophistication in recent Russian *Orthopoxvirus* research.

A paper from Australia on aerosols of *Orthopoxviruses* – here using vaccinia virus as a surrogate for variola virus – had six authors, all with Russian names (99). The scientists used a Collison nebulizer to aerosolize droplets of vaccinia virus; virus recovery was then detected by sampling the aerosolized material using a prototype sampler. Although we made no effort to investigate former work by these scientists (such would be beyond the scope of this project), the publication is a reminder that a number of Russian scientists with expertise in *Orthopoxviruses* may have moved to other countries and continued to work in their field of expertise.

Human tumor necrosis factor (TNF), despite its name, is an inflammatory cytokine that protects against many of the adverse consequences of viral infections. TNF is targeted by *Orthopoxviruses*, including variola virus, as part of the viral strategy of interfering with host anti-viral immune responses. Russian scientists used standard molecular genetics techniques to produce recombinant proteins from variola virus, monkeypox virus, and cowpox virus, with particular emphasis on immune modulatory proteins (100, 101). Such work could provide additional insights into how *Orthopoxviruses* evade anti-viral immune responses, and also yield potential approaches to altering a less pathogenic virus into one more pathogenic. Russian scientists found substantial homology among aligned sequences of the anti-TNF proteins of variola virus and the anti-TNF proteins of both

cowpox viruses and monkeypox viruses (102). Not reported are results of similar studies with camelpox virus, of interest due to the homology between variola virus and camelpox virus (90). Scientists interested in such a comparison could perform analyses from published sequence data. Functional studies of the TNF-binding proteins showed some differences between the variola-derived protein and those from the other *Orthopoxviruses* studied (102). However, more work would need to be done to understand the genetic and functional differences in detail.

Poxviruses show an impressive homology of promoter sequences between genera. As a result, it is possible to heat-kill a pathogenic poxvirus but "rescue" it by adding a live second non-pathogenic virus which would provide the enzymes needed for transcription and perhaps for other functions. In such a circumstance, it may be possible to retain the DNA template of the heat-killed pathogenic virus, which could possibly be used to make more of the infectious pathogenic virus (103). This process of *non-genetic activation* potentially could be used to convert a non-infectious smallpox virus into an infectious one. It also might be used to obtain a virulent variola virus from its full genomic DNA, for example, as carried by Russian scientists in plasmids, though it would take a fairly sophisticated molecular virology laboratory to carry out such a procedure (15, 105). This phenomenon also has been called heterologous reactivation, a process whereby a cell infected with one poxvirus can produce infectious virus from the pure DNA of a second poxvirus (106). Such a method could allow resurrection of a no longer extant poxvirus from its known DNA sequence after synthesis of the DNA itself. In such a manner, DNA derived from the sequence of variola virus might possibly be used to grow variola virus that contained a complement of proteins required for the first rounds of replication followed by packaging of fully functional variola viruses. As a result, destruction of all variola virus stocks would not necessarily mean that the earth would thereafter forever be free of the virus.

Indeed, it has been suggested by Chinese scientists that global warming might release smallpox virus from sites in the tundra or in glaciers so as to induce a new smallpox pandemic (107). Although variola viruses are rather stable in the environment, the chance that a natural epidemic might occur in this fashion is conceivable but unlikely. It remains possible, however, that such a source of variola virus might be available even after all laboratory samples are destroyed. Viable variola virus might be obtained from a natural depot and then grown for various purposes. In this regard, Russian scientists have pointed out that variola viruses can live for long periods of time – a hundred years or more – in the tissues of corpses in permafrost areas or after burial in crypts (5, 108).

Homologous recombination is a frequent occurrence in poxvirus-infected cells (109-111). Thus, simultaneous infection of a cell with two different *Orthopoxviruses* can lead to emergence of new *Orthopoxviruses*, some of which might be especially virulent for humans. Indeed, studies by Norwegian scientists demonstrated *in vitro* recombination between a vaccinia virus strain and a cowpox virus strain (109). It is possible that recombination between, for example, a camelpox virus and a vaccinia virus might yield recombinant viruses of concern. In one study, Canadian scientists found that the vaccinia virus DNA polymerase has certain properties that allow recombination (112). If such a

recombination occurred, the resulting modification of the *Orthopoxvirus* – even without change in its virulence – might make initial detection difficult if based upon restriction endonuclease patterns (110).

In populations immune to smallpox, as in the past in the United States, a mild infection in a partially immune individual would pose little risk to contacts. The situation is very different for a non-immune population. If a partially immune person were to be infected with variola virus, the residual immunity could reduce the severity of disease in that individual, but he or she might not effectively clear the undetected virus. This scenario would allow him or her to shed the virus while not incapacitated, thereby potentially infecting many contacts (113).

This possibility is relevant to certain terrorist scenarios that have been advanced. For example, it has been suggested that fanatics might allow themselves to be purposely infected with the variola virus and then walk through US airports to infect people around them. Smallpox experts have suggested that such a scenario is unlikely as people with contagious smallpox would be rendered too ill by the disease to carry out such an assignment. However, if a person had minimal immunity (e.g., from a vaccination many years before), he or she might be able to secrete virus and yet be physically strong enough to try to spread disease in public places.

Russian scientists working at VECTOR, a former BW research center, have published several scenarios of potential future outbreaks of smallpox (36). They have emphasized mathematical modeling of the spread of smallpox under various situations of bioterrorism (36). These authors suggest a need for communications among responders and decision-makers; requirements for medical personnel, hospital beds, and medical materials; and estimates of the rate of spread of the disease. Other Russian scientists at the same institution published mathematical models of the spread of microbiological agents of concern through the atmosphere, including modeling of movement through indoor air (37). Such work has influenced the design of buildings in the region and the modification of ventilation systems (37).

Scientists at another former BW research center in Russia – Gamaleya Research Institute of Epidemiology and Microbiology – described a scenario in which variola virus was released in a city of a million people (38). The analysis included mathematical modeling of the spread of disease and potential consequences depending upon the reactions of public health authorities (38).

Between scenario development, computer modeling of the spread of smallpox, new oral vaccination methods, an initiative to immunize against smallpox, and development of human antibodies specific for variola virus, it appears that Russia is taking extensive measures to deal with a potential outbreak of smallpox. Russia has involved their Center for Military and Technical Problems of Biological Protection at the Ministry of Defense, as well as other agencies, in the mathematical modeling-computer simulation (39).

In contrast to the situation in Russia and the former Soviet Union, which had an intensive research effort into *Orthopoxvirus* genetics, China has shown little evidence in the open literature of a large, long-standing laboratory program into *Poxviruses*. However, in recent years Chinese scientists have manipulated poxviruses, and China's general programs in molecular virology appear to have narrowed the prior gap between itself and Russia. Indeed, Chinese scientists have analyzed immune modulating poxvirus proteins (114, 115), once a subject of substantial research by Soviet scientists. Chinese scientists have cloned an anti-apoptosis virulence factor of goatpox virus and analyzed it (116). Such an experiment raises the possibility of Chinese scientists inserting various virulence factors into an *Orthopoxvirus* readily able to infect humans. Indeed, the Chinese have separately engineered a human interleukin gene and an interferon gene into an *Orthopoxvirus* (75, 76). Although these last experiments are of no direct concern, the approach and the techniques could possibly lead to creation of an *Orthopoxvirus* virulent for humans. In this regard, some of China's capabilities in molecular biology generally and specific analyses of certain molecules are relevant to understanding virulence mechanisms of *Orthopoxviruses* (117).

China has published its awareness of the possible bioterrorism use of variola virus (9-11) and expressly indicated that cultures of the variola virus stored in the former states of the Soviet Union could possibly have been commandeered by terrorists considering the employment problems after the disintegration of the Soviet Union and the departure of many Soviet scientists (118, 119). To protect itself from such a possible event, China has been exploring the use of various viruses as vaccines, including cowpox strains (9).

Over the years, the effectiveness of vaccination against smallpox with vaccinia virus reduced the impetus to develop drugs effective against the virus for use either in prophylaxis or in therapy. However, with the eradication of smallpox as a naturally occurring disease and suspension of routine public health vaccination programs, a gap was increasingly appreciated. Basically, it was recognized that, despite its effectiveness in protecting against the dreaded smallpox, vaccination with the old vaccinia viruses used in the past for smallpox vaccines caused unacceptable adverse side effects in too many recipients to justify its use in large populations against a disease that no longer existed in nature (61, 120-123). However, in a desire to avoid the potential of being unprepared for a possible terrorist attack with variola virus (the causative agent of smallpox) or a related virus, many countries have tried to develop either less toxic vaccines or new drugs that might serve to prevent or treat smallpox or a related disease caused by another *Orthopoxvirus*. As a result of these new efforts, developmental work has covered (i) vaccines with fewer adverse events, and (ii) testing of known drugs and new compounds for potential use as anti-*Orthopoxvirus* agents. This type of work is ongoing in several countries and has yielded some promising results (24-26, 122, 124-131). A DNA molecule containing immune-stimulatory CpG sequences also may assist in the therapy of *Orthopoxvirus* infections (124). However, just as antibiotic-resistant bacteria have become a problem, the potential for development of anti-viral resistant *Orthopoxviruses* also is a concern. Indeed, it was found that vaccinia virus strains became resistant to the anti-viral drug cidofovir during serial passage in Vero cells in culture (130). Cross-resistance was found for compounds related to cidofovir but not to unrelated nucleosides

(130). This result also raises the issue of potential purposeful development of a virulent *Orthopoxvirus* pathogenic for humans that might be resistant to the most promising anti-smallpox drugs currently under serious study.

As time passes, new issues not considered in the past become important. For example, bovine spongiform encephalopathy (Mad Cow Disease) may be passed to people via animal proteins that bear the inducing prions. To prevent this from occurring via animal proteins contaminating a smallpox vaccine, there is a push in some countries to use vaccine produced in tissue culture (132).

In the event of a new poxvirus outbreak in people, responses can include mass vaccination or vaccination and quarantine of index cases and their contacts. The details of expected public health responses to a poxvirus outbreak provide data for mathematical models. However, a predicted and required rapid response may not occur in time for contact (ring) vaccination plus quarantine to contain an outbreak.

In addition to its use as a vaccine for smallpox, during the past 25 years vaccinia virus has been employed for a variety of secondary purposes, such as vectors for antigens or genes (65-67, 70, 72-75, 80, 81, 83-88, 133-149, 150-189). The virus has been used to transfer genes in medical research – in cancer treatment, for example. Such work depends upon the ease in growing vaccinia virus and its stability during storage as well as the ability of vaccinia virus to carry substantial quantities of foreign DNA and yet continue to function normally (133). In addition, many countries have been using vaccinia viruses to carry other antigens in immunization efforts. This last use is based in part on the ability of vaccinia virus to stimulate host dendritic cells, which are critical to initiating a strong host immune response (138, 163, 190).

Conclusions

Even if US and Russian stocks of *variola virus* are destroyed, the threat of smallpox will not necessarily have been eliminated. The virus may be sequestered elsewhere in hidden collections or could be recovered from burial sites of humans who died of smallpox (108).

Several *Orthopoxviruses* currently replicating in nature are able to infect humans: monkeypox, cowpox, vaccinia, and others. These could evolve to be worse human pathogens. Such a process, however, might take many decades. These other *Orthopoxviruses* also could be genetically engineered in the laboratory into more virulent human pathogens by techniques known to virologists around the world.

Currently available vaccines will probably protect against the naturally occurring viruses, such as monkeypox, cowpox, and vaccinia viruses as they evolve, but they might not protect against certain genetically engineered viruses.

There has been concern regarding the level of persistent immunity against smallpox in individuals vaccinated decades ago. In addition, a certain level of confusion has been introduced by variable results regarding the degree of residual immunity from different studies in various countries. Some studies have shown substantial antibody levels against vaccinia virus in a number of individuals, whereas other studies describe less robust immunity. In one series of studies of interferon-gamma-producing memory T cells, only 20% of people vaccinated 13-25 years previously had adequate responses (191). However, in another study of residual immunity against smallpox, the presence of a vaccination scar plus a history of vaccination within 20-30 years correlated with good T cell reactivity (192). Overall, among people vaccinated against smallpox in the distant past, it is likely that (i) some such individuals are no longer protected, (ii) some are partially protected and would therefore get mild disease if infected with the variola virus, and (iii) some are completely protected. The great majority of Americans born since 1972 would not have been immunized and would not be protected

Short of a recurrence of smallpox or the emergence of a new aggressive *Orthopoxvirus*, Americans who are not immune should not be given the old vaccinia smallpox vaccine. That historical vaccine is too toxic, inducing unacceptable adverse reactions in many people (61, 120-123). A newer vaccine with far fewer side effects might be acceptable as a relatively routine immunogen should one be required.

Publications Reviewed

More than 2,000 papers on poxviruses from the worldwide open literature published between 2002 and the latter part of 2007 were reviewed for this report, as well as a few earlier papers (most of those were from Russia). Papers selected for analysis primarily dealt with genetics, molecular genetic manipulations, virulence mechanisms and modifications of those, and approaches to therapy.

Background Information on Smallpox and Related Poxviruses

General

The *Poxviridae* family contains large double-stranded DNA viruses that encode between 150 and 300 proteins. The portion of this family that infects vertebrates, called *Chordopoxvirinae*, encompasses eight genera. The ***Orthopoxvirus* genus** – with which we are especially concerned herein – contains viruses with a linear double-stranded DNA genome of between ~170,000 and ~220,000 base pairs and includes the following species: (i) **Variola virus (the causative agent of human smallpox)**, (ii) Monkeypox virus (strains of which can induce a generalized infection in humans), (iii) Cowpox virus (which can infect humans), (iv) Vaccinia virus (used to vaccinate humans against smallpox, and which can infect humans). [Buffalopox virus is a variant of vaccinia virus that can infect humans (193, 194). There are several Brazilian variants of vaccinia virus circulating in the wild, some of which have caused disease in people (195). Rabbitpox

virus is believed to be a vaccinia variant that did not directly evolve from any sequenced vaccinia virus strain (196). Although it causes severe disease in rabbits, that virus does not affect humans.] (v) Camelpox virus, which is closely related to variola virus and which has occasionally caused relatively mild disease in humans, (vi) volepox virus, (vii) skunkpox virus, (viii) racoonpox virus (197), (ix) Tatera virus (gerbilpox virus), (x) mousepox virus (called ectromelia virus), and (xi) Uasin Gishu (horsepox) disease virus (198). Racoonpox, volepox, and skunkpox viruses are North American viruses.

Variola virus, the causative agent of smallpox, is closely **related by DNA homology studies to camelpox virus and Tatera (gerbilpox) virus and also is related to skunkpox and racoonpox viruses**.

Overall, there are *Orthopoxviruses* that can cause generalized disease in humans, others that typically lead only to limited skin infections, and some that have not infected humans.

Smallpox Epidemiological Issues

Smallpox (caused by variola virus) afflicted humans for thousands of years. When introduced into completely non-immune populations, such as the Native Americans of South America and North America, the death toll was especially severe. This is an issue today as routine vaccination against smallpox has been suspended worldwide since about 1982 with the worldwide elimination of the naturally occurring disease in 1977. As a result of this suspension of vaccination against smallpox, most of the US population is now susceptible to smallpox disease should it be reintroduced by accident. Moreover, there has been concern about the use of variola virus as a biological warfare (BW) or bioterrorism agent (2, 199).

After smallpox disease had been eradicated worldwide, the World Health Organization moved to eliminate the causative virus from all laboratories and strain collections. Variola viruses were first to be confined to two centers, one in the United States and one in Russia, and those two strain collections later destroyed. However, these viral collections have not yet been eliminated. Moreover, there is some concern that other variola stocks might exist in countries that may not have destroyed their variola virus collections as planned by the WHO. The United States has argued that additional research was needed to better understand the virus in order to develop suitable anti-smallpox drugs in case the disease recurred for whatever reason.

Clinical Features of Smallpox

Person-to-person spread of smallpox is primarily by respiratory droplets and less often by viral transmission via scab material. The virus particles in the aerosol droplets of an infected individual originate in erupting lesions on the pharynx, larynx, tongue, and

uvula, and less often from the trachea and bronchi. Such viral secretion starts slightly before or at the same time as the skin rash and persists for approximately one week.

After a person is newly infected with variola virus, the incubation period prior to the development of clinical smallpox is approximately 10-14 days. The disease is characterized by the sudden onset of headache, backache, fever, and malaise. The typical rash occurs three to four days later: the first appearance is on the person's buccal and pharyngeal mucosae, followed quickly by eruptions on the face, forearms, and hands. Initially, the lesions are macules (flat), followed by firm papules (heaped up), vesicles (fluid filled), and finally pustules (with white blood cell accumulation). Eight to ten days after onset of the rash, the pustules dry up. Healing leaves pockmark scars, especially on the face. In smallpox, the rash typically occurs distally (i) on the face to a greater extent than on the trunk, (ii) on the legs and forearms more intensely than the arms and thighs. In contrast, chickenpox is distinguished by a central (trunk, arms, and thighs) rather than a peripheral pattern of rash. Rash on the hands and/or feet should point to smallpox rather than chickenpox.

One clinical type of smallpox, caused by variola major, had a case fatality rate of ~30%, whereas another clinical type, variola minor, had a case fatality rate of only 1%. These clinical features correlate with genetic differences between the strains.

Vaccination with vaccinia virus vaccine strains is protective against smallpox for some five to ten years, with waning immunity thereafter. Even some immunity may render the disease much milder than would be the case without any prior immunizations. Nonetheless, as most people in the United States were last vaccinated before 1971 or not at all, the majority of the population is considered to lack immunity to smallpox.

Laboratory Diagnosis of Smallpox and Other Orthopoxviruses

Prior to the elimination of smallpox as a naturally occurring disease, the clinical features were usually sufficient to make an accurate diagnosis of smallpox. For confirmation, virus-containing fluids from skin vesicles or scabs were tested on the chorioallantoic membranes of chick embryos, and the patterns and colors of the pocks that formed on those membranes were used to make a diagnosis of the virus species. Electron microscopic features also were examined for laboratory confirmation. Since then, PCR assays have been developed to detect *Orthopoxviruses* generically and also to distinguish among the eleven species of *Orthopoxviruses* (95, 97, 110, 200-220).

The *Orthopoxviruses* make an HA protein, whereas no other poxviruses make an HA protein. This has allowed development of a PCR reaction to distinguish *Orthopoxviruses* from other poxviruses.

PCR of the HA gene can distinguish the various species in the *Orthopoxvirus* genus. Such assays can even distinguish a variola major strain from a variola minor strain (221,

222). However, some studies suggest that certain cowpox virus strains have an HA gene that is detected by variola primers and probes. Therefore, where possible, viral genome sequencing should be performed to provide a definitive method of species and strain identification.

There is only about a 75% genomic DNA homology between Old World *Orthopoxviruses* and *Orthopoxviruses* isolated in North America. In contrast, among the Old World *Orthopoxviruses*, the sequence homology is ~ 95%. When *Orthopoxviruses* are divided into two groups, Group I includes Old World vaccinia virus (along with its variants: rabbitpox and buffalopox), monkeypox viruses, and the closely related ectromelia virus (mousepox). Group II includes variola virus (the causative agent of smallpox) and the closely related camelpox and tatera gerbilpox viruses**.** Also included are skunkpox and racoonpox viruses. Each of those two groups of viruses has a genetic relatedness to a different group of cowpox viruses, in keeping with the idea that an ancient cowpox virus may have given rise to all of the *Orthopoxviruses* (223).

Monkeypox

With the eradication of smallpox as a disease in nature, monkeypox virus has become the naturally occurring *Orthopoxvirus* most able to cause systemic disease in immunologically intact people. Human disease caused by the monkeypox virus is found in the tropical rainforests of western and central Africa. Some monkeypox strains cause less severe disease in humans than others (224).

The incubation period for monkeypox is approximately 12 days, followed by marked malaise with severe muscle aches, headaches, and cough. The rash and clinical features of human monkeypox are indistinguishable from smallpox except that monkeypox is characterized by more marked lymph node enlargement (89). Person-to-person spread of monkeypox is less common than for smallpox, from rare up to 30% of human cases in different outbreaks (89, 225, 226). People most often become infected with monkeypox virus by handling infected animals (wherein the virus is thought to enter via cuts or abrasions) or from eating infected monkeys (bushmeat) or infected squirrels.

Although monkeys develop monkeypox disease, and many have antibodies to the virus, the animal reservoir for monkeypox virus is believed to be squirrels. The PCR test can diagnose monkeypox virus as an *Orthopoxvirus*, and specific primers can distinguish monkeypox virus from other *Orthopoxviruses*. Another laboratory method is the inoculation of virus-containing fluids from skin vesicles or scabs onto test chorioallantoic membranes of chick embryos. The appearance of the pocks that form on those membranes is used to make a species diagnosis. In addition, the broad susceptibility of various test animals to the virus assists in the identification of monkeypox virus.

Purposeful monkeypox virus infection of monkeys demonstrated that post-infection smallpox vaccination was not as good as anti-viral drug therapy (227). Whether this will hold for humans remains to be determined.

An outbreak of monkeypox occurred in 2003 in the United States as a result of the importation of a virus-carrying Gambian pouched giant rat. The African rodent infected pet prairie dogs (intermediate hosts) in the US facility, which led to disease in 72 people (half confirmed in the laboratory) some of whom became seriously ill (224, 228-230). Fortunately, this infection was from a variant strain of monkeypox virus that causes relatively mild disease and shows limited human-to-human transmission (224, 229). Infections occurred largely from contact, many presumably through skin lesions (230). Nonetheless, that outbreak raised the specter of a serious monkeypox outbreak outside of Africa and even the possibility that monkeypox might begin to fill the niche that smallpox had occupied for thousands of years.

Differences in the genetics of the different strains of monkeypox virus were found by Russian scientists to explain the differences in virulence of the strains (231). The Central African monkeypox virus has factors preventing host immune responses to infection with the virus, making it especially virulent. The West African strain has a deletion of one such gene, which encodes the complement-binding protein that interferes with host-antiviral responses. Without that gene product, the virus is more readily cleared by the host and it causes less severe disease (89, 231). Fortunately, it was the milder form that was imported into the United States in 2003.

Genetic studies suggest that monkeypox arose independently of smallpox (19, 89). The central regions of their genomes are 94-96% homologous; however, the outer, species-specific, regions are less homologous (19, 89, 226).

Genetic comparisons from a US-Russian collaboration suggest that the monkeypox virus would not easily transform into a virus like variola virus under natural selection in the wild in the near term, though that could possibly occur over the stretch of future history (19).

Cowpox

Cowpox viruses have large genomes and are the *Orthopoxvirus* with the most complete set of genes, suggesting to scientists that they are the likely precursors of the other extant *Orthopoxviruses*. For example, several genes in cowpox viruses are found in only fragmentary forms in the other *Orthopoxviruses* (223, 232). Further analysis indicates that there are two genetically separable groups of cowpox viruses that may have diverged evolutionarily long ago (232). It is thought that each of those two cowpox virus groups is the precursor of the two major groups of *Orthopoxviruses*.

As a disease of cows, cowpox is manifested as ulcers on the teats, which leads to pustular lesions on the hands of people milking the cows. More than two centuries ago, such lesions on the hands of milkmaids were found to protect against the subsequent development of smallpox. However, the animal reservoirs of cowpox viruses are not cows but several kinds of rodents (bank voles, field voles, and wood mice in the UK and

elsewhere in Europe, susliks and gerbils in Turkmenistan, root voles in Russia, and lemmings in Norway) (233-235). Such animals can infect additional mammals, including domestic cats and zoo animals (236, 237). Humans infected with cowpox viruses can have virus circulating in their blood streams even if the disease is relatively limited (238). Human cowpox disease currently is contracted primarily from infected domestic cats (which had contracted the virus from rodents) (203, 239-244), although dogs (245) and horses (246) also may be implicated.

Cowpox in people has an incubation period of about seven days, followed by the sudden appearance of fever, malaise, headaches, and muscle aches, sometimes accompanied by nausea and vomiting. Skin lesions are most common on the hands and face. Local lymphadenopathy is marked. Healing of the skin takes place over several weeks, leaving a large depressed scar. The disease can be fatal.

It has been found that cowpox virus interferes with induction of host nuclear factor-*kappa*B, a global immune regulatory transcription factor (247). This effect could contribute to the failure of host immune responses to adequately clear the virus. It is presumed that related mechanisms would occur in variola virus infection.

From an evolutionary perspective, a fit *Orthopoxvirus* would have the greatest host range (be able to infect many different animal species), be transmitted effectively from animal to animal in a not very dense population, would replicate rapidly and effectively in each host, and would balance its pathogenicity with its ability to reproduce and be passed on to other hosts. From this perspective, cowpox virus in perhaps the most evolutionarily fit *Orthopoxvirus* and variola virus the least fit (248).

Vaccinia

Vaccinia virus, the vaccine strain for preventing smallpox, was the first animal virus seen microscopically, grown in tissue culture, and purified. However, vaccinia viruses have a poorly understood origin. They may have originated from a now extinct *Orthopoxvirus* of horses that caused a disease called “grease” (249) or perhaps from a recombinant of cowpox virus and this grease-causing virus. The vaccine virus was passaged many times, which undoubtedly introduced several changes into the viral genome. Indeed, the resultant vaccine virus variants were selected for those strains that induced less pain, fever, and other complications after vaccination of people.

As a result of this process, vaccinia viruses were kept as laboratory strains for long periods of time. This led to a belief by many scientists that there was no natural reservoir for vaccinia (250). However, the genome of horsepox virus isolated from Mongolian horses points to a vaccinia-like structure (249). This finding suggests the possibility of a relationship between horsepox virus and the ancestral strain originally used for vaccination and from which vaccinia virus may have been derived.

Several variants of vaccinia virus have escaped into animal populations and now circulate in nature. Some of these vaccinia variants can cause disease in humans. For example, buffalopox in India is due to vaccinia virus variants, of which several have been distinguished (193, 194). A buffalopox virus can cause skin disease in humans who touch the infected teats of milking water buffalo or dairy cows, but systemic symptoms are usually quite mild or absent. Buffalopox viruses also cause oropharyngeal lesions in people who drink unpasteurized milk carrying the virus (193, 228). Rabbitpoxvirus, which infects rabbits, also is a vaccinia virus variant, but one that does not usually infect people and which may not have derived from a vaccine strain (196).

A number of strains of vaccinia virus circulate in various animals in Brazil (250-256), strains that show divergent evolution but which appears to have derived from vaccine strains. Many of these strains have infected people.

In addition to its role as a vaccine for smallpox, vaccinia virus has been used as a vector via genetic engineering to deliver genes and/or to function as a carrier in immunizations against a variety of diseases (including HIV and other infections). Vaccinia is especially useful in this regard because it can be genetically engineered to accept a large amount of additional DNA without loss of its ability to replicate.

Camelpox virus

Camelpox virus is the virus most closely related to variola virus, the cause of smallpox (90). Variola virus and camelpox viruses produce the same small white pocks on the chorioallantoic membranes of fertilized hens' eggs and have a similar upper temperature limit for growth of such pocks. Neither of the two viruses can grow in rabbit skin. Genetic analyses show that (i) the sequences of the two viruses are closer than either is to any other virus, (ii) the arrangements of the protein coding regions are most similar to each other, (iii) the protein sequences themselves are most closely related, and (iv) the nature and structure of the terminal repeats are most similar between variola and camelpox viruses (90). The observation that camelpox is endemic in camels has raised the concern that a camelpox virus isolated from animals in one or more regions might somehow be converted into a lethal, variola-like human pathogen.

The susceptibility of various potential host species to camelpox virus has been incompletely studied. Rabbits, guinea pigs, and three-day old chickens are reportedly susceptible (257); however, studies with the M-96 strain of camelpox virus could not confirm those findings (257), suggesting possible variability from strain to strain.

Russian scientists also have compared the genetic organization of camelpox virus with those of other *Orthopoxviruses*. Their studies suggest that variola virus and camelpox viruses diverged approximately 6,000 years ago (96, 248), whereas modern *Orthopoxvirus* species derived from a precursor virus ~12,000-14,000 years ago (89).

Other poxviruses that can sometimes cause disease in humans

Parapoxviruses cause disease in sheep and goats, called "orf," characterized by papules and vesicles on the mouth and nostrils of the animals (258). Such disease in lambs can cause lesions on udders of nursing ewes and vice versa. The diseases are found in Africa (especially north of the equator), western Asia, India, China, and Bangladesh (258). Although orf once had been thought to be caused by the same *capripoxvirus* in both sheep and goats, recent work suggests that the sheep and goat diseases may be caused by viruses that have genetic differences that allow molecular tests to discriminate them (258, 259). In fact, some of the capripoxviruses are specific for sheep (sheep pox viruses), some are specific for goats (goat pox viruses), and some may infect either sheep or goats (a less discriminating form of sheep pox virus) (258). Recombinants also occur between various strains of sheep pox virus. This type of disease can occur in cattle, caused by (i) bovine papular stomatitis virus or (ii) *Pseudocowpox* virus. Humans touching the teats during milking contract the virus and develop local lesions on the hands. The human lesions resolve within six weeks. There is also a seal *parapoxvirus* (*Sealpoxvirus*) that rarely has infected humans. Occasionally, poxvirus infections of humans can be misdiagnosed as an *Orthopoxvirus* when the infecting agent is a *Parapoxvirus* (260).

Yatapoxviruses

Tanapoxvirus and Yaba monkey tumor poxvirus are *Yatapoxviruses* that occasionally infect humans. The animal reservoirs in the wild of these two viruses are unknown as is the mode of transmission to humans.

Molluscum Contagiosum

A skin disease of humans in which the skin seems to be heaped up is caused by the Molluscum Contagiosum virus. Although especially found in children, in recent years this disorder has occurred in many AIDS patients. Indeed, Molluscum Contagiosum has become a marker for sexually transmitted diseases generally.

Structure of Orthopoxviruses

These are big, brick-shaped viruses, able to be seen on light microscopy. The outer part of the viruses consists of a membrane composed of tube-like lipoprotein subunits. This membrane encloses a dumbbell-shaped core and two "lateral bodies" (261). The core consists of the viral double-stranded (ds) DNA and associated proteins. Cowpox virus is the largest of the ***Orthopoxviruses*** at ~220,000 base pairs. Variola viruses run at approximately 186,000 base pairs of dsDNA. The DNA has a C+G content of ~34%. These viruses have been sequenced (95-97, 221). Among the genes found in common, variola, vaccinia, and cowpox viruses have >90% genetic identity.

Each end of the virus DNA has an inverted terminal repeat (ITR) that is A+T rich and incompletely base paired, giving it a hairpin loop that connects the two DNA strands (sometimes called cross-linking). Genes encoding species-non-specific "housekeeping" proteins involved in replication and other critical viral functions are found in the central 90-100 kb of the *Orthopoxvirus* genome and are highly conserved (89, 262). In contrast, genes encoding species-specific proteins (such as those that interfere with the immune functions of a particular host species) are located peripherally (toward the ends of the DNA (89, 262).

Poxvirus Replication

Infection of a host cell by an *Orthopoxvirus* leads to major cytopathic effects, changes in membrane permeability, and inhibition of cellular DNA, RNA, and protein synthesis as the virus essentially takes control of such functions and directs them to producing viral molecules.

Poxvirus replication occurs completely in the cytoplasm, a feature seen in only one other type of virus: African swine fever virus. An *Orthopoxvirus* gene product associates with host cell membranes, including those of the endoplasmic reticulum, in helping to determine the cytoplasmic sites of viral replication (263).

Some *Orthopoxviruses*, such as vaccinia viruses, can enter nearly all kinds of cell lines and infect them. As a result, such viruses appear not to take advantage of a specific receptor for viral attachment and entry but may use a virus-binding site that allows viral attachment but not entry. Entry occurs when the cell-associated enveloped virion (CEV) fuses with the host cell membrane with or without an endocytic process, and the viral core is released into the cytoplasm (264). The genomes of poxviruses encode dozens of enzymes needed for transcription and replication of the viral genome. Some of these factors are pre-formed and present in the virion, including DNA-dependent RNA polymerase, capping enzyme, methylating enzymes, and transcription factor.

Pre-formed viral transcriptase initiates transcription [copying of viral DNA to make messenger RNA (mRNA)]. As a result, functional capped, polyadenylated, and methylated viral mRNAs are produced within minutes after infection of a host cell. Polypeptides produced by translation (making a polypeptide from the viral mRNA) act to finish uncoating the viral core. Transcription of ~100 "early" genes occurs prior to the initiation of viral DNA synthesis. These "early" synthesized proteins include enzymes needed for replication of the viral DNA, such as DNA polymerase and thymidine kinase. Indeed, half of the vaccinia virus genome is transcribed even before replication of viral genomic DNA – an impressive "hitting the road running" adaptation.

This "early" process is followed by a shift in gene expression to "intermediate" gene transcription at approximately 100 minutes after infection of the host cell, and then by "late" gene transcription, which starts about 140 minutes after infection of the cell. These intermediate and late events happen via the action of specific viral proteins on promoter

sequences and may be controlled by positive and negative regulatory proteins. Most intermediate and late viral mRNA molecules have a 5' poly (A) tail, whereas most early mRNA do not. Viral DNA replication is thought to begin near the ends of the viral DNA genome.

Some viral proteins are secreted by infected cells. These proteins promote infection. Many are proteins that interfere with host immune responses, such as cytokine analogues and cytokine receptor analogues with functions relating to host interferons, interleukins, complement, chemokines, and tumor necrosis factor (89, 115, 228, 265-274). Included are (i) immunosubversive proteins that mimic host ligands, (ii) regulatory proteins ("*virokines*") such as homologues of epidermal growth factor, a complement regulatory protein, and virokines that confer resistance to host interferon, and (iii) "*viroreceptors*" that are homologues of cellular receptors but have lost their transmembrane anchors and signaling functions; these bind and sequester important immune regulatory molecules, preventing the molecules from reaching the appropriate host cell receptors. Included in this last category are molecules that bind tumor necrosis factor (TNF)-*alpha*, interleukin-1-*beta*, interleukin 18, CC chemokines, interferon *alpha/beta*, and interferon *gamma* (89).

Because of their importance for virulence, *Orthopoxviruses* produce two intracellular proteins and two extracellular proteins that interfere with the actions of host interferons (89). Besides interferons, tumor necrosis factors are the other critical immune actors in viral infections. Despite its name, human TNF is an inflammatory cytokine that protects against many adverse consequences of viral infections. TNF is targeted by *Orthopoxviruses*, including variola virus, as part of the viral strategy of interfering with host anti-viral immune responses (89). Russian scientists lined up the sequence of the anti-TNF protein of variola virus with anti-TNF proteins of cowpox viruses and monkeypox viruses and found substantial homology (102). Not reported are results of similar studies with camelpox virus, of interest because of the known homology between variola virus and camelpox virus. Functional studies of the TNF-binding proteins showed some differences between the variola-derived protein and those from the other *Orthopoxviruses* studied (102).

It has been found that cowpox virus and vaccinia virus produce factors that interfere with induction of host nuclear factor-*kappa*B, a global immune regulatory transcription factor (247, 268-270). This effect contributes to the failure of host immune responses to adequately clear the virus. It is presumed that related mechanisms would occur in variola virus infection. The more these virus-induced modulations are analyzed, the more viral proteins appear to be involved and the more complicated the interactions (268-270, 275).

The host complement system contributes to anti-viral responses. However, one of the vaccinia secreted proteins inhibits both the classical and alternative complement pathways of the host – this occurs through binding and inactivation of C4b and C3b complement molecules (231, 266, 267, 272, 276, 277). The variola virus complement control protein is more potent than that from vaccinia (272).

The *Orthopoxvirus* replication process within the infected cell uses specific areas of the cytoplasm for assembly of daughter viruses, sites that can be visualized by electron microscopy. A complete early transcription system is produced late in the infection of a cell and packaged within the core of infectious poxvirus particles. This allows the daughter virions to immediately begin their life cycles as soon as they enter other, non-infected host cells. Most of the daughter viral particles are released without envelopes via cell disruption. A smaller number are released with envelopes by exocytosis. Although both enveloped and non-enveloped viruses are infectious, the enveloped viruses are more infectious (278).

Homologous recombination is a frequent occurrence in poxvirus-infected cells. Thus simultaneous infection of a cell with two different poxviruses can lead to emergence of new poxviruses with recombined genes (232).

Treatment and Vaccination – a 21st Century Issue

Vaccination with live vaccinia virus was effective in protecting against smallpox; however, the vaccine did cause adverse reactions, including generalized vaccinia disease in some people with eczema or immune deficiencies (61, 120-122, 279). Historically, approximately 30 people per million vaccinated had a life-threatening reaction to the vaccine and one to two people per million vaccinated died with one preparation (120, 122) and there were up to eight deaths per million people vaccinated with another vaccine preparation (120). Adverse reactions included eczema vaccinatum, progressive vaccinia, post-vaccinial encephalitis, fetal vaccinia, vaccinia keratitis, myopericarditis, and inadvertent transfer of virus from a newly vaccinated person to others (122, 123). Nowadays, up to 30% of people have contraindications to vaccination with live vaccinia virus, including cancer, HIV, heart problems, skin diseases, treatment with immunosuppressive medicines, pregnancy, or household contacts with such contraindications.

Despite these adverse events from vaccination against smallpox, over the years, the effectiveness of vaccination with vaccinia virus in preventing smallpox disease reduced the impetus to develop drugs effective against variola virus for use either in prophylaxis or in therapy of smallpox. However, with the eradication of smallpox as a naturally occurring disease and suspension of routine public health vaccination programs, a gap was increasingly appreciated. Despite its ability to protect against *Orthopoxviruses*, vaccination with vaccinia virus caused unacceptable side effects in too many recipients to justify its use in large populations against a disease that no longer existed in nature. In a desire to avoid the potential of being unprepared for a possible terrorist attack with variola virus (the causative agent of smallpox) or a related virus, many countries have tried to develop either less toxic vaccines or new drugs that might serve to prevent or treat smallpox or a related disease caused by a naturally occurring or genetically engineered *Orthopoxvirus*. As a result of these new efforts, developmental work has covered (i) vaccines with fewer adverse events, and (ii) testing of known drugs and new

compounds for potential use as anti-*Orthopoxvirus* agents. This work is ongoing in several countries and has yielded some promising results.

There is extensive immunological cross-reactivity and cross-protection among the *Poxviridae* strains within a given genus but little among strains in different genera. As a result, monkeypox virus, vaccinia virus, and cowpox virus protect against smallpox. This type of cross-protection has encouraged scientists to try several potential approaches to new vaccines. There is special interest in recombinant vaccines that might avoid the side effects seen in some people vaccinated with live vaccinia virus.

An effective immune response against a poxvirus includes both T cell-mediated immunity and antibody production. T lymphocytes in the form of cytotoxic T cells eliminate variola virus-infected cells even prior to viral replication, limiting an infection. Antibodies able to bind enveloped and non-enveloped virions protect against re-infection.

The standard US vaccine against smallpox was for many years a lyophilized live vaccinia virus preparation called Dryvax. A single vaccination protected against smallpox disease for about five years. A second vaccination protected for at least 10 years thereafter. Because of the long incubation period for smallpox, it is possible to effectively vaccinate someone even after he or she is exposed to the variola virus. Vaccination in the first four days after exposure can often prevent disease. Vaccine given on day 5 or 6 may ameliorate the disease to some extent (280). Patients with eczema may develop widespread disease from vaccination. Such complications have been treated successfully in the past with vaccinia immune globulin; however, such globulin is now in limited supply. The optimal time for harvesting such immune globulin is found to be 14 days after a revaccination (281).

For decades, Russian scientists have been evaluating an oral form of live vaccinia virus as a human vaccine. This oral vaccine has shown far fewer adverse reactions in humans than the standard skin scarification method with no diminution in immune protection (27). Russian workers also have combined the oral smallpox vaccine with an oral Hepatitis B vaccine (282).

A strain of vaccinia virus that had been used as a smallpox vaccine was found by Japanese scientists to become more virulent after growth in culture due to a mutation in a single gene (283). That gene could be eliminated without loss of protective immunity (283). The resulting strain was as protective as the US Dryvax strain and yet much less virulent. Additional work on the vaccine strain lacking the complicating [B5R] gene has produced what appears to be a much safer vaccine, one that might be sufficiently devoid of serious adverse effects to be considered for large population groups (21-23). This vaccine protects early in infection by inducing cell-mediated immunity more than antibodies (284). However, British scientists argue that retention of the B5 protein is critical for a smallpox vaccine because that immunogen is what is recognized by important antibodies (285). This is an issue in the trade off between good immunization and adverse events.

Monkeypox virus infection of monkeys demonstrated that post-infection vaccination was not as good as anti-viral drug therapy (227). Whether this tendency will hold for humans remains to be determined.

A DNA polymerase inhibitor used in HIV infections called cidofovir has anti-*Orthopoxvirus* activity; however, that drug can be nephrotoxic. Some related compounds may be given orally and cause less toxicity (127, 228). Ribavirin, another antiviral drug, may also have anti-*Orthopoxvirus* effects.

Selected Open Source Publications by Country

Russia

During Soviet times, Russia carried out intensive studies of *Orthopoxviruses.* For many years, the research into variola genetics was part of a vigorous military program. However, the extent of that work may not be reflected in the open literature. As a result of its long commitment to understanding variola virus genetics, Russia has substantial experience to draw upon in terms of manipulating *Orthopoxviruses*. That represents an important basis of concern for the United States, Canada, Mexico, Europe, Japan, Australia, and other nations in terms of the potential for creation of genetically manipulated *Orthopoxviruses* pathogenic for humans. Such a concern is superimposed upon that relating to the possibility that some Russian variola or variola-like viral stocks may have fallen into the hands of terrorists.

Although the present report focuses on open source publications in the years 2002-2007, we are aware of several earlier Russian papers. Russian scientists reported the complete sequence of a virulent variola virus in 1992 (89). A 1992 publication reported the cloning of the growth factor gene of vaccinia virus (286). Studies from 1993 compared variola and vaccinia viruses (287, 288). Another 1993 paper provided information on a study of genetic engineering of vaccinia virus, which may have been part of a systematic analysis of *Orothopoxvirus* genes (289). A 1996 paper also reported on genetic engineering of *Orthopoxviruses* by adding genetic material (290). Some of these experiments analyzed virulence mechanisms of the virus (288). Papers from 1995 compared in detail the sequences of a variola virus strain that had caused an outbreak of smallpox in India in 1967 with a variola strain that had caused an outbreak in Bangladesh in 1975 (93, 291). Further studies compared the sequences with vaccinia virus sequences and analyzed their genomic organization (291, 292). Alignment of the DNA genomes showed a 99.3% homology. Of 200 protein sequences, 122 were identical, 42 had a substitution in a single amino acid, 11 had changes in two amino acids, and the other 25 had bigger differences. Such studies can assist in forensic analyses. It also was noted that a region of conservation in variola virus is variable in vaccinia viruses (93).

The extensive work on *Orthopoxviruses* gave Russian scientists insights into the mechanisms by which such viruses elude host immune responses (89, 98, 232, 293). Such information could have provided them with clues to genetic alterations of *Orthopoxviruses* that might make the viruses more virulent to humans. This concern has been magnified by the reported increase in virulence seen after vaccinia virus (20) and then mousepox virus were manipulated so as to carry the gene for mouse interleukin 4 (IL4) (42). Russians have published papers about their genetic manipulations of *Orthopoxviruses*, so far emphasizing changes that make the viruses less pathogenic (294-297); however, some of the same techniques could possibly lead to more virulent cowpox or vaccinia viruses. There is concern that an available *Orthopoxvirus* engineered to carry the human IL4 gene might be especially virulent.

Overall, it appears that as modern molecular genetics techniques have become more sophisticated and disseminated worldwide, whatever advantages the Russians may once have had may have been reduced or even lost. Indeed, studies from Australia and France appear to be as sophisticated as the recent Russian studies or more so. Papers from Brazil may be in the same class as those of the Russians. During the 1990s, Russia experienced a marked reduction in funding for biological research, which led to their failure to develop a modern biotechnology industry and the desertion of many biological scientists. It also has prevented Russia from maintaining and building on its prior gains in viral research. Shortage of funds has led to publications describing experiments that used old-fashioned equipment and a limited supply of reagents. This, along with internal political factors, often leads to publications in Russian journals not readily available to the international scientific community.

Also to be considered are the efforts at collaboration between US and Russian laboratories in recent years. This has included funding of Russian scientists by the US Civilian Research and Development Foundation (219) and the International Science and Technology Center (89), in part in an effort to employ Russian experts who might otherwise take their expertise and skills to other countries. These collaborations have included genetic studies (19, 94, 298). The full sequence of a monkeypox virus (197 kb) was compared with that of a variola strain (~11-12 kb smaller). The central regions, containing genes necessary for survival and replication in a host cell, were 96.3% identical. However, there were considerable differences in the ends of the genomes where species-specific factors are encoded (19). Comparisons of variola virus strains (94) grouped the viruses into three phylogenetic clusters: (i) consisting of three sub-groups – China-Korea-Sumatra; Japan; Central Asia-Near East, (ii) an Asian cluster restricted to an India-Nepal group and a Bangladesh group, and (iii) West African and South American stains, perhaps connected by the early slave trade (94).

Russian scientists also have independently published results of sequencing of a number of *Orthopoxviruses* (95-97). Those studies have provided insights into pathogenetic mechanisms of infection with such viruses and the evolution of *Orthopoxviruses* (95, 97). It remains unclear how much of this work was performed recently and how much was carried out many years ago, was not published at the time, and has now been updated and published as new work.

A recent Russian paper (4) stated that "smallpox virus is kept in laboratories of [*some* or *certain*] countries." (Brackets and italics added). This wording may hint at Russian knowledge of stocks in countries other than the United States and Russia, but could refer only to the United States and Russia. Another Russian paper states that according to US experts: "At least 10 countries have such collections and this, through accidental leaks (spillages), could possibly endanger the entire world health protection system." (5).

Studies of the ability of different variola strains to infect different classes of host cells indicate that the envelope structure of the virus may contribute importantly to virulence (299).

Russian scientists have developed PCR and microarray assays for the *Orthopoxvirus* genus and for specific *Orthopoxviruses*, such as variola virus (the causative agent of smallpox), monkeypox virus, vaccinia virus, cowpox virus, camelpox virus, and others (95, 97, 110, 215-220, 298, 300-306). Such assays distinguish the varicella-zoster virus (the causative agent of chickenpox and a virus unrelated to *Orthopoxviruses*). This is important because clinical chickenpox may be confused with the clinical presentation of various *Orthopoxviruses*.

Russian scientists at VECTOR created sets of plasmids with each set carrying the complete genome of one of thirteen strains of variola virus (15-17). Such plasmids could potentially be used to reconstitute a viable variola virus or used to create a modified virus with unusual properties. Such plasmids could possibly be used to separately transport non-infectious parts of variola virus to be reassembled after the travel was completed.

In preparation for the plasmid work, it was found that 32 of 54 variola virus isolates were viable and 29 suitable for the relevant long PCR (307).

A phage combinatorial library was screened for scFv single-chain human antibodies reactive with variola, vaccinia, and cowpox viruses (308-317). The purpose was to identify and then grow large quantities of antibodies in case of a new outbreak of *Orthopoxvirus* disease in humans (308, 318). Russian scientists also have produced full-length recombinant antibodies (319).

Between scenario development, computer modeling of the spread of smallpox, new oral vaccination methods, and development of human antibodies specific for variola virus, it appears that Russia is taking extensive measures to deal with a new outbreak of smallpox.

Human tumor necrosis factor (TNF) is an inflammatory, anti-viral cytokine that protects mammalian hosts against many of the adverse consequences of viral infections. TNF is targeted by *Orthopoxviruses*, including variola virus, as part of the viral strategy of interfering with host anti-viral immune responses (265, 320). Russian scientists used standard molecular genetics techniques to produce recombinant proteins from variola virus, monkeypox virus, and cowpox virus, especially immune modulatory proteins (98, 100, 101, 320). They have analyzed in detail the TNF-binding proteins (98, 101, 265). Such work could provide insights into how *Orthopoxviruses* evade anti-viral immune responses and also yield potential approaches to alter a less pathogenic virus to make it more pathogenic. Russian scientists aligned the sequence of the CrmB protein, the anti-TNF protein of variola virus, with anti-TNF proteins of cowpox viruses and monkeypox viruses and found substantial homology (~90%), but with a number of differences as well, with the lowest homology (85%) between variola virus and monkeypox virus proteins (102, 265). Not reported are results of similar studies with camelpox virus, of interest because of the known strong homology between variola virus and camelpox virus. However, scientists interested in such a comparison could probably perform the analyses from published sequence data. Functional studies of the TNF-binding proteins showed some differences between the variola-derived protein and those from the other

Orthopoxviruses studied (102), with the suggestion of species-specific effects (265). More work would need to be done to understand the genetic and functional differences in detail. Nonetheless, the Russian scientists did find that recombinant variola virus CrmB worked effectively *in vitro* (321) and decreased the mortality of mice in an endotoxic shock experiment (322). These last studies demonstrate the functional significance of variola proteins that interfere with host immune responses. It also points to a dual use situation in which variola proteins may be used for clinical therapeutic purposes.

Studies related to the TNF protein of *Orthopoxviruses* involved interferon-gamma-binding proteins of these viruses, proteins that also facilitate viral virulence by interfering with anti-viral host responses (323). New therapeutic uses of the proteins are being sought (323).

Mousepox, caused by ectromelia virus, was ameliorated by treatment with interferon-gamma or tumor necrosis factor alpha or both (324). These results may be relevant to treatment of *Orthopoxvirus* infections of humans.

A Russian study showed that ectromelia virus (mousepox) and cowpox virus (also thought to be carried by rodents) differed in terms of their pathological effects on mice. The cowpox virus strain used caused a localized type of infection, whereas ectromelia produced a more disseminated infection (325). However, it is possible that other cowpox virus strains might have different effects. Indeed, sequencing of cowpox virus strains has indicated that cowpox virus has the most complete set of *Orthopoxvirus* genes and that cowpox strains vary to a greater extent than strains from other *Orthopoxviruses* (223). This work points to cowpox viruses as the precursors of variola virus, vaccinia virus, and ectromelia virus because several genes in cowpox virus are found in only fragmentary forms in the other *Orthopoxviruses* (223).

Morphological aspects of various *Orthopoxviruses* were reported with an emphasis on their appearance while growing on chick embryos (326) as well as in various cell types (327).

Russian scientists have reported that variola virus can live for long periods of time in the tissues of corpses, a hundred years or more in permafrost areas or after burial in crypts (5, 108).

Russian scientists, like scientists in many other countries, have used vaccinia virus for several purposes: (i) immunizing against antigens of other pathogens, (ii) anti-cancer treatments, and (iii) gene therapy (65, 66, 67).

Intranasal infection of mice with a cowpox virus strain was studied (328). Intranasal studies have similar implications to aerosol studies. In this circumstance, virus replication in the respiratory tract was found and analyzed.

Aerosol studies were carried out to optimize detection of an airborne release of *Orthopoxviruses*, in this case using vaccinia virus (215, 216). It was found that use of

PCR in the test process markedly increased the sensitivity and decreased the time of analysis.

Russian scientists working at a former BW research center (VECTOR) have published scenarios of potential future outbreaks of smallpox (36). They have emphasized mathematical modeling of the spread of smallpox under various bioterrorism scenarios (36). Requirements for medical personnel, hospital beds, and medical materials; estimates of the rate of spread of the disease; and the need for communications among responders and decision-makers are included. Other scientists at the same Russian institution published mathematical models of the spread through the atmosphere of microbiological agents of concern. These models included dissemination of microbial agents through indoor air (37). Such work has influenced the design of buildings and has led to the modification of ventilation systems (37).

Russian scientists at another former BW research center – Gamaleya Research Institute of Epidemiology and Microbiology – described a scenario involving the release of variola virus (the causative agent of smallpox) in a city of one million people (38). Analyses of this release included mathematical modeling of the spread of disease, with different consequences depending upon the reactions of the public health sector (38).

Russia has involved their Center for Military and Technical Problems of Biological Protection at the Ministry of Defense in the mathematical modeling-computer simulation activity (39). Included in their analyses are releases in (i) closed systems, such as subways, (ii) open air places where people congregate, and (iii) other sites (39).

The genes of Kelch-like proteins from variola virus, monkeypox virus, cowpox virus, and vaccinia virus were compared. These gene sequences were found only in variable terminal regions of the *Orthopoxvirus* genomes (329).

Russian scientists have been evaluating an oral form of live vaccinia virus as a human vaccine for decades. This oral vaccine showed far fewer adverse reactions than did the standard skin scarification method in more than 6,000 human subjects (27, 248, 330, 331). Moreover, protective immune responses after oral vaccination were as good as after scarification, both in terms of antibody titers and cell-mediated immunity. This oral vaccine also showed protection of people from smallpox in Ethiopia in 1972-1973 (27). The Russians believe that oral immunization is much safer than the older scarification method (248, 332) and should be the preferred method in the event of a new outbreak of smallpox (4). It was noted that children playing with ampules of the vaccinia virus could and did become ill (333).

Russian scientists also have developed an oral vaccine against both smallpox and hepatitis B (334-340), apparently based on the oral vaccine with which they had experience (4, 27). Such work is consistent with their encouragement of the simultaneous administration of vaccines against multiple agents, a strategy designed to save money and reduce the number of required medical visits. These studies suggested

that prime and boost with just the oral vaccine may be sufficient to induce a good anti-smallpox response (334).

A 2005 paper described oral immunization of rabbits with vaccinia virus (341). It would seem that a 35-year experience with oral immunization of people would not be followed by rabbit studies, but that animal experiments would precede human use. It is possible that the reported rabbit studies filled some gap in the human trials. Alternatively, the rabbit studies may have been carried out in the distant past and only recently published.

Russian scientists have also developed a DNA vaccine against variola virus, so far tested in mice (26).

Several studies show that cidofivir and related compounds are effective against *Orthopoxviruses*. Ribavirin appears to have some activity against *Orthopoxviruses*. Combinations of anti-virals may be useful (342). Additional anti-smallpox drugs are under study (343); however, most do not have activity against *Orthopoxviruses* (344).

Many rat monoclonal antibodies to ectromelia virus cross-reacted with certain specific determinants on vaccinia virus, cowpox virus, and smallpox virus (345). Only those antibodies reactive with a 14 kDa polypeptide were cross-neutralizing of *Orthopoxviruses* (345). In addition, some ectromelia virus-specific antibodies were found. Related antibody studies also were reported in another paper (346).

Molecular genetics techniques were used to distinguish vaccine and epidemic strains of sheeppox virus and to distinguish sheeppox and goatpox viruses (259, 347). Other studies established techniques for producing and measuring antibodies to such viruses (347-349).

Genetic variability among strains of monkeypox virus was found by Russian scientists to explain the differences in virulence of the strains (231). The Central African monkeypox virus has virulence factors preventing host immune responses to infection with the virus. The West African strain has a deletion of one such gene (the complement binding protein), preventing production of the gene product that interferes with host-antiviral responses. Without that gene product, the virus is more readily handled by the host, and it causes less severe disease (231). Fortunately, it was the milder form that was imported into the United States in 2003. Had the more virulent form been imported into the United States, much more severe disease might have occurred.

China

In contrast to the situation in Russia and the former Soviet Union, which had an intensive research effort into *Orthopoxvirus* genetics, based upon the open source literature, China has not had a large, long-standing laboratory program into *Orthopoxviruses*. However, in recent years Chinese scientists have manipulated such viruses; moreover, its general programs in molecular virology have narrowed the prior gap between itself and Russia.

Chinese scientists have reviewed the world literature on *Orthopoxvirus* genomic organization (350) and have studied individual genes (351, 352). They also have analyzed immune-modulating poxvirus proteins (114, 115), a subject of great interest to Soviet scientists. Chinese scientists have cloned an anti-apoptosis virulence factor of goatpox virus and analyzed it (116). Such an experiment raises the possibility of Chinese scientists inserting various virulence factors into an *Orthopoxvirus* readily able to infect humans. Of special note is the genetic engineering of a human interleukin gene and an interferon gene into *Orthopoxviruses* (75, 76). Although such experiments are of no direct concern, the approach and the techniques could possibly lead to creation by genetic engineering of an *Orthopoxvirus* virulent for humans.

China has published its concern about the potential bioterrorism use of variola virus (9-11, 353). The Chinese expressly indicate that cultures of the variola virus stored in the former states of the Soviet Union could possibly have been commandeered by terrorists, in light of the employment problems after the disintegration of the Soviet Union and the departure of many Soviet scientists (118, 119). Moreover, Chinese scientists see variola virus as easy to grow and aerosolize (353). Chinese papers even discuss creation of an *Orthopoxvirus* that is engineered to carry genes from Ebola virus or from monkeypox virus (353). To protect itself from such a possible event, China has been exploring the use of various viruses as vaccines, including cowpox strains (9), and has analyzed the potency and host range of vaccinia strains (354-356). Chinese scientists also have studied anti-smallpox immunoglobulin (357).

China has used vaccinia virus as a carrier for various antigens in immunization efforts (70, 72-75, 80, 81, 83, 139-162,). It has used vaccinia virus for presenting Nipah virus antigens to the immune system (140, 358). Such studies used altered codons to make them most compatible with mammalian immune systems (74, 79, 140, 150). Chinese scientists also used vaccinia for attempts to immunize against HIV (70-83, 359). In addition to its utility as a vaccine vector, vaccinia virus has been employed as a vector of gene therapy, especially for malignancies (68, 69).

Assays for detection of *Orthopoxviruses* have been developed, including PCR reactions and microarrays (207-214, 360-365). Included are assays to distinguish variola virus from monkeypox virus (360, 361).

Scientists in China have compared the genomes of various *Orthopoxviruses* and have constructed a phylogenetic tree (366).

Chinese scientists have genetically engineered poxviruses. They have studied capripox virus (49, 208, 367-374) and have modified a capripoxvirus (capripoxviruses cause a pox disease in goats and sheep as well as lumpy skin disease in bovines, and they occasionally infect humans) for use in immunization strategies (208, 367-373, 375). The studies included deletion of a virulence gene. In principle, addition rather than deletion of virulence genes also could be performed. In other studies, vaccinia virus was modified to make it safer by removing a gene encoding an interferon-*gamma* binding protein (352).

Of note, capripoxvirus was found to carry a gene encoding a protein able to bind IL18 as did ectromelia virus and vacccinia virus (49). The binding of IL18 increases virulence.

Studies of vaccinia virus have included production of recombinant viruses (355). Although less virulent recombinants may be useful for vaccine strains, the more virulent ones may be problematic in the future. An enhancer element appears to play a role in gene expression (376).

Methods for the large-scale production of smallpox vaccine viruses have been established (377). Such methods could also be used to grow up large numbers of other poxviruses. The method involved growth in 15 liter bottles, rather than in large bioreactors. Survival of vaccine virus was greater at -20 degrees C than at room temperature (377).

Chinese scientists studied the host range of vaccinia virus with the apparent goal of using vaccinia vaccine in horses and other equines (77). However, any studies of host range should be scrutinized as there are concerns that the information might be used to expand the host range of animal poxviruses to humans.

China's interest in mousepox (caused by ectromelia virus) is consistent with the occurrence of that disease in institutional animal facilities, as occurs in the West (378). However, studies of mousepox virus may have application to variola studies.

Chinese scientists have reviewed the non-Chinese research into monkeypox viruses (379-382) and have expressed concern about the possible introduction of monkeypox into China.

One paper suggested that global warming might release variola virus from sites in the tundra or in glaciers so as to induce a new smallpox pandemic (107). Although the variola virus is rather stable in the environment, this suggestion seems unlikely. It remains possible, however, that viable variola virus might be obtained in such a manner and then grown up, which would be a serious concern.

Like scientists in several other countries, scientists in China have established mathematical models of the spread of disease (40). In one smallpox model, 100 infected people would produce 16,530 infected people in 30 days (40).

Australia

Overall, Australian scientists have produced several interesting and important papers on poxviruses, some of which describe sophisticated scientific methods and thinking.

Perhaps the most important research has to do with IL4 effects on mousepox virus disease. In studies with far-reaching implications, Australian scientists engineered the gene encoding the mouse IL4 gene into mousepox virus (also called ectromelia virus) and discovered that the mousepox virus bearing the IL4 gene had very different properties

than ordinary mousepox virus (41, 42). **The IL4-bearing mousepox virus acted as a super-pathogen**. IL4 expression caused disease in mouse strains normally genetically resistant to mousepox, leading to a high mortality (41, 42). IL4 expression also led to a delay in vaccinia virus clearance from target organs and death of mice infected with vaccinia virus bearing the mouse IL4 gene (20, 41). The physiological basis of these findings is understood. It is known that Th1-type T cells are critical for the cell-mediated immunity and interferon gamma that clear virus-infected cells and that IL4 suppresses the development of such cells (41). Therefore, the marked over-expression of IL4 caused by the modified viruses prevents viral clearance. It was also noted that the antiviral drug cidofovir failed to protect mice from the mousepox virus that bore the mouse IL4 gene (46).

This finding with the IL4-modified mousepox virus has raised the real possibility that a genetically engineered *Orthpoxvirus* able to infect humans, such as vaccinia virus or cowpox virus, might be made more virulent for humans by similar genetic engineering with the human IL4 gene. In the Australian study, the mouse IL4 gene was incorporated into the mousepox virus and the genetically modified virus administered to laboratory mice. That single genetic alteration (addition of the gene encoding IL4) led to fatal disease in all strains of mice tested, even in mice normally resistant to mousepox. Of perhaps even greater concern, the IL4-bearing mousepox virus overcame standard immunizations that normally protect the mice from mousepox disease. That is, mice vaccinated against mousepox resisted the usual mousepox virus but came down with fatal disease when they were given the IL4-containing mousepox virus (42). The failure of immunization to protect suggests that the IL4-bearing virus interfered with the expression of memory immune responses. This set of results indicates that it is entirely possible that genetic modification of an *Orthopoxvirus* that causes mild human disease (vaccinia, monkeypox, cowpox, camelpox, etc.) might give rise to an *Orthopoxvirus* that is as lethal as variola virus. Thus, one might not even need variola virus to cause a serious *Orthopoxvirus* outbreak – modification of a readily available *Orthopoxvirus* might serve that purpose. Moreover, such an engineered *Orthopoxvirus* might even be virulent in people immunized against smallpox. Therefore, experiments in which immune mediators are engineered into *Orthopoxviruses* should be viewed with suspicion.

Mouse strains (C57BL6) that produce lots of IFN gamma, IL2, and TNF produce cytotoxic T cells and are relatively resistant to mousepox infection; in contrast, mouse strains that primarily generate an IL4 response (BALB/c and A/J) fail to produce enough cytotoxic T cells (383). The mice that fail to make cytotoxic T cells succumb to the infection, whereas those that do make cytotoxic T cells survive a mousepox infection (383). Some of the interferon effects may be mediated by interferon inducible chemokines (384).

Australian scientists found that antibody is critical for control of secondary poxvirus infections and that interferon is critical in primary infections (385). The ability of poxviruses to produce factors that inhibit interferon function worsens disease.

Australia is concerned about smallpox stocks being in the possession of "rogue states" or bioterrorists, raising the issue of the need for vaccination against smallpox (12).

Live vaccines for smallpox require refrigerated transport and storage (29), suggesting a requirement for alternate approaches. One such alternative might be peptide vaccines; however, they may not provide long-term immunity and might not induce immunity in people with certain MHC types (29). In addition, somewhat toxic adjuvants may be required to induce an adequate immune response with peptide antigens. A lipid-core peptide system that incorporates a lipid adjuvant, carrier, and peptide antigen may overcome these drawbacks (28, 29). Moreover, particles with both immunological adjuvant and carrier functions activate host dendritic cells, which is a critical feature for working very well as immunogens in vaccines (28, 138).

Like scientists in many countries, Australian scientists are developing vaccinia virus as a carrier for other antigens for treating varied infections or cancer (163-168, 386).

Tumor necrosis factor (TNF) production by host cells is critical to recovery from poxvirus infections (387). Studies of mice infected with ectromelia virus (mousepox virus) show that TNF regulates the traffic of immune cells into infected tissues. However, poxviruses produce factors (homologues of receptors for TNF) that bind TNF and, thereby, reduce the effects of host TNF responses (387).

Studies in mice showed that vaccinia virus had developed ways to prevent the action of interferon-inducible T cell alpha chemoattractant, which causes inflammation after other viral infections (388). This factor is induced by interferon-gamma. It is likely that vaccinia-produced proteins that bind to interferon gamma are responsible for this observation.

T cell responses were studied after people were vaccinated with vaccinia virus (389). CD4+, CD38+, CCR5+ T cells and interferon-gamma producing cells peaked at 14 days (389). Neutralizing antibodies increased between days 14 and 21.

Papers from Australia on aerosols of *Orthopoxviruses* – here using vaccinia virus as a surrogate for variola virus – had six authors, all with Russian names (99, 390). This work is a reminder that Russian scientists with expertise in *Orthopoxviruses* may have moved to other countries and continued to work in the field. The studies themselves involved development of personalized samplers for measuring exposure to *Orthopoxviruses* released into the atmosphere.

Like many other countries, Australia has developed an emergency plan to deal with a re-introduction of smallpox (30). Mathematical models of such an event indicate that rapid identification of cases, combined with quarantine and prevention of contact would be effective (30). They suggest that prior mass vaccination would be inefficient (30). However, this conclusion is predicated on a rapid identification of a small number of affected individuals, which may not be possible in the United States.

New Zealand

New Zealand scientists, like those from other countries, have used vaccinia virus as a vector for other antigens (169).

Taiwan

Residual immunity against smallpox was measured by assessing T cell reactivity to vaccinia virus (192). The presence of a vaccination scar plus a history of vaccination within 20-30 years correlated with good T cell reactivity.

Aspects of vaccinia virus morphogenesis were studied with an emphasis on temperature sensitivity of various critical proteins (391).

Vaccinia virus entry into host cells was found to involve plasma membrane lipid rafts (392).

Scientists in Tawian have used vaccinia virus to carry other antigens in cancer treatment and in immunizations against other infections (393).

Japan

Japanese scientists and public health officials are concerned about a possible re-emergence of smallpox and have undertaken plans for responses to such an event (394, 395).

Japan's stocks of vaccine for smallpox were destroyed in 1997 because the vaccine had lost its potency (13). A new vaccine was developed because of fears of bioterrorism (13, 14). Nonetheless, some humoral immunity appears to persist even in people last vaccinated nearly 30 years in the past (396, 397), especially in those individuals with at least three vaccinations (31, 398).

A strain of vaccinia virus that had been used as a smallpox vaccine was found to become more virulent after growth in culture due to a mutation in a single gene (283). That gene could be eliminated without loss of protective immunity (283). The resulting strain was as protective as the US Dryvax strain and yet much less virulent. Additional work on the vaccine strain lacking the complicating (B5R) gene has produced what appears to be a much safer vaccine, one that might be sufficiently devoid of serious adverse effects to be considered for large population groups (21-23, 399). This vaccine appears to protect early in infection by inducing cell-mediated immunity more than antibodies (284). British scientists also have been studying B5R effects in a vaccine and believe that the B5 is needed for good immune responses (400).

Mathematical modeling of a potential new outbreak of smallpox suggests that mass vaccination is preferable to vaccination of only case contacts when the Ro (the measure of the number of individuals infected by a single individual with the disease) is high and when public health intervention is delayed (14). Thirty million to fifty-six million doses of vaccine would be needed for such mass vaccination (14, 31).

Japanese scientists reviewed the literature on monkeypox and appeared to be concerned about possible importation of that disease (401-403). More recently, they have carried out experiments in monkeys with the virus obtained from human patients with monkeypox disease in Zaire (Congo Basin virus) and Liberia (West-African virus) (404). They found – as had others previously – that the Congo Basin type of monkeypox is more severe than the West African type of monkeypox (404).

Taterapox virus is closely related to camelpox virus and variola virus. Sequences in the mammalian poxviruses have derived from reptilian sequences (405).

The same vaccine strain of vaccinia virus could give different results in a plaque reduction neutralization assay if different cell types were used for plaquing (406).

Japanese scientists created vaccinia viruses that bore human cytokine genes by genetic engineering (59). The cytokine genes included those encoding IL12, IL23, and IL17 (59). Although none of these constructs are of direct concern, any engineering of interleukin genes into a poxvirus pathogenic for humans is worrisome. These studies further found that anti-IL17 antibodies could make vaccinia virus more virulent in mice (59). IL23 also contributes to resistance to vaccinia virus (59). Therefore, interfering with IL17 and/or IL23 could make an *Orthopoxvirus* infection of humans more severe.

Scientists in many countries have used vaccinia virus as a vector for other antigens or for gene delivery. Japanese scientists also have so employed vaccinia virus (170, 171, 407-413). In addition, Japanese scientists have been developing detection methods for various *Orthopoxviruses* (414).

South Korea

A South Korean study found persistent humoral (antibody) immunity but not cell-mediated immunity against vaccinia virus in people vaccinated more than 25 years previously (415). For short-term effects, dilution of the vaccine did not impair development of T cell mediated immunity (416). Previously immunized people had higher antibody titers than naïve individuals prior to revaccination, as would be expected, and had fewer adverse events (417).

South Korean scientists found that vaccinia-related kinase 3 suppresses extracellular signal-regulated kinase (ERK) activity through direct binding to a vaccinia H1-related MKP, which inactivates the ERK (418). Indeed, vaccinia regulates the level of

transcription factors (419). An efficient protein expression system was used to express vaccinia-related kinase 1 (420, 421).

Vaccinia virus was used to carry other genes in cancer therapeutics (422). A Yatapoxvirus protein that interferes with host interferon was found to complex with the so-called Z form of DNA (423).

A mathematical model of a smallpox epidemic was developed to evaluate spread in a Korean city and the roles of possible interventions (32).

Thailand

Scientists in Thailand developed mathematical models of the epidemiological outcome of a smallpox outbreak in *Japan* (33). They found that residual immunity in a portion of the population would markedly reduce the magnitude of the crisis (33).

India

Several outbreaks of buffalopox were reported (424). The genetics of buffalopox virus isolates were studied. These viruses were found to be highly homologous to vaccinia virus (194) but must be distingushed from pseudocowpox, which also occurs (425).

Indian scientists have been analyzing the detailed mechanisms by which the *Orthopoxvirus* complement-binding protein interferes with host complement activity (266, 267).

Scientists in India like those in many other countries have been using vaccinia virus to carry genes for genetic therapy or for immunization against the encoded antigens (426).

Kazakhstan

A strain of camelpox virus (M-96) was administered to a variety of farm animals and poultry; however, none became infected (257). It is possible that this strain was less virulent than other strains of camelpox virus because previous reports had indicated that three-day-old chicks, guinea pigs, and rabbits were susceptible to camelpox (257).

Turkey

Concerned about biological warfare, Turkey has been developing educational and laboratory plans in case of an emergency (427).

Israel

Responses to revaccination of Israeli adults were measured (428). Only 56-61% of the people had successful revaccination rates; however, the levels of pre-existing antibodies inversely correlated with the "take." Therefore, a greater percentage may actually be protected. Consistent with that result, the greater the time since last vaccination, the greater the rate of conversion. The optimal time for harvesting vaccinia immune globulin was found to be 14 days after a revaccination (281).

Virus encoded growth factor plays an important role in the *Orthopoxvirus* life cycle (429).

Lithuania

Public health responses are being planned to counter bioterrorism events, including one with smallpox, (430).

Czech Republic

Vaccinia virus was found to induce apoptosis in a macrophage cell line through the action of early gene expression (431). That result is superficially at variance with the finding of an apoptosis inhibitor in vaccinia virus (432).

In a collaborative study between Czech and US scientists, it was found that vaccinia virus strains became resistant to the anti-viral drug cidofovir during serial passage in Vero cells in culture (130). Cross-resistance was found for compounds related to cidofovir but not to unrelated nucleosides (130). This result raises the issue of potential purposeful development of a virulent *Orthopoxvirus* pathogenic for humans that might be resistant to the most promising anti-smallpox drugs currently under serious study.

A paper from 1983 indicated that only a few camel herdsmen developed skin lesions from camelpox and that worries about human camelpox are overblown (433).

Slovakia

The soluble vaccinia virus-produced protein B18R contributes to virulence by binding Type I interferons produced by the host (434).

Poland

Polish scientists studied certain cellular transcription factors after vaccinia virus infection of human macrophages (435). The experiments provide information on mechanisms of

protein transport in human macrophages during infection with vaccinia virus. The studies are reasonably sophisticated. In somewhat related experiments, Polish scientists studied expression and localization of heat shock proteins after macrophages were infected with vaccinia virus (436). The data suggest that vaccinia viruses use several cellular anti-apoptotic mechanisms to prolong the viability of the macrophages that harbor the virus and, thereby, facilitate viral replication. Studies of host Bcl-2 expression after vaccinia virus infection of human monocytes has shed further light on the anti-apoptosis processes (437).

A vaccinia virus protein able to bind IL1 showed immunosuppressive activity (271). This is one of the immune mechanisms used by *Orthopoxviruses* to resist immune clearance by the host.

It was found that treatment of infected cells with IL1, IL6, and corticosteroids might delay virally induced inhibition of host protein synthesis (438). However, it is not clear whether such approaches would be clinically useful.

Serbia

Serbia is concerned about possible bioterrorism with smallpox (439).

Bulgaria

Bulgarian scientists found a synergistic therapeutic benefit of cidofovir and idoxuridine (IUdR) on vaccinia virus replication in cell cultures of chick embryo fibroblasts (440). However, it is unclear if this is applicable to treatment of people because IUdR is toxic to humans.

Spain

Spanish scientists like those of many other nations have used vaccinia viruses as vectors to deliver antigens or genes (172-179, 441-447). They also have developed detection methods for *Orthopoxviruses* (448).

Recombinant vaccinia viruses were constructed by homologous recombination (449). In addition, Spanish scientists genetically engineered vaccinia virus to carry the genes encoding IL12 and IL18. Experiments with these genetically engineered viruses demonstrated that IL12 and IL18 cytokines contribute to the clearance of the virus (48). This clearance was also facilitated by the involvement of host NK cells and T cells (48). However, it is of concern that cytokines are being engineered into vaccinia viruses and perhaps other *Orthopoxviruses* because some of the cytokines may yield a much more virulent *Orthopoxvirus* as when the mouse IL4 gene was engineered into ectromelia

(mousepox) virus (42). Moreover, interfering with such a cytokine as IL18 might increase virulence.

Scientists in Spain are analyzing events after vaccinia virus infection of human cell lines in an attempt to better understand *Orthopoxvirus* infections, including variola (450). Spanish studies demonstrated that the B1R kinase of vaccinia virus is an early gene required for viral DNA synthesis. The BIR kinase hyper-phosphorylates host p53 molecules which leads to degradation of the p53, contributing to viral control of the host cell machinery (451). They also discovered that a Wiskott-Aldrich protein played a role in vaccinia virus infections (451).

Spanish scientists studied the interactions of vaccinia with host cell membrane proteins (453). Vaccinia virus was found to infect host monocytes most readily, followed by B cells and NK cells (454). Very few T cells were infected.

Italy

Like scientists from many other countries, Italian scientists have used vaccinia virus to carry antigens for cancer therapy or for immunization against various infectious diseases (455-457).

T cell responses after smallpox vaccination were monitored by flow cytometry (458).

Switzerland

CpG motifs packaged into viral-like particles induced protective cytotoxic T cell responses (459). Vaccinia virus has been used to carry gene segments in cancer therapy (460, 461).

France

Residual immunity to variola virus may be inadequate to protect the civilian population. In studies of interferon-gamma-producing memory T cells, only 20% of people vaccinated 13-25 years previously had adequate responses (191).

French health officials have been considering potential responses to the threat of smallpox reintroduction. If vaccination with live vaccinia virus of the populace were to be resumed, in addition to the right vaccinia preparation, a good stock of vaccinia immune globulin would be needed (462). Work on such a supply of immune globulin is underway in France. Also, a national network of suitable laboratories for anti-vaccinia immunoglobulin is being established (463). Decontamination methods would be needed as well. Toward this end, a non-corrosive method for decontaminating *Orthopoxvirus*-harboring environmental areas and surfaces has been established (464).

New vaccine production capabilities are being developed (465). Non-replicating vaccinia viruses are being studied for potential utility in humans as smallpox vaccines (466). Such studies are first carried out in a mouse cowpox model (25). Although potentially less likely to cause serious side effects in people, the non-replicating vaccinia strains conferred *less* long-term immunity than classical replicating vaccinia vaccine strains.

French scientists also have been searching for anti-variola drugs. Delivery systems may be critical for the use of such drugs because of the poor *in vivo* conversion of nucleoside analogue drugs to their active triphosphate nucleotide forms (467). In an attempt to overcome this problem, the pro-drugs were encapsulated into a poly(iso-butylcyanoacrylate) aqueous core nanocapsule. However, this did not work as well for these mononucleotides as had been found for oligonucleotides (467). Additional work on formulation will be required. One possible approach is putting the nucleoside analogues, such as cidofovir, into lipids for better delivery (468). Preliminary studies suggest that this may be a useful approach (468). However, issues of drug resistance also have arisen (469).

Detection methods for variola virus and viruses causing diseases resembling smallpox have been developed in France (470, 471).

France, like many other countries, has been using vaccinia viruses to carry other antigens (134-138, 472-474). This ability is based in part on the stimulation of host dendritic cells, which are critical to initiating a strong host immune response (138).

Mathematical models were developed to assist in the management of a potential biological attack with variola virus in France (34). One such model dealt with a smallpox outbreak in a city of two million people (34). Three critical parameters were the basic reproduction number (Ro, the number of cases a single affected individual would cause), time to intervention by the public health authorities, and the proportion of contacts of cases that were traced and vaccinated (34).

French scientists engineered human IL2 into vaccinia virus to study feline fibrosarcomas (47). Although this work is directed at treatment of human cancers (47), such studies are a concern and would be a greater one if an adversary had performed such a study, because certain interleukins have the potential to make poxviruses, including vaccinia virus, monkeypox virus, and variola virus, much more virulent than they otherwise would be.

Using bioinformatics, a French scientist studied the evasion of Toll-like receptors by vaccinia virus by production of a protein that acts on the TLR-IL1 receptor (475). This receptor undergoes dimerization and then complexing to other molecules.

French scientists have studied the *Orthopoxvirus* DNA repair protein called uracil-DNA glycosylase (UNG), which is important in viral pathogensis (476). This enzyme preferentially cuts out uracils when opposite a cytosine (476). Future research will be

directed at developing inhibitors to UNG. In other experiments, French scientists have analyzed the immune response to cowpox virus in mice (477).

Germany

Codon usage affects the rate of expression of genes, especially genetically engineered genes (478). Low translation initiation and elongation rates are especially associated with insufficient amounts of a corresponding tRNA and to a changed start codon context (478). The nucleotide composition of the mRNA (codon usage) was found to limit gene expression; however, a vaccinia virus polymerase hybrid system improved such expression (478). The results suggested that codon usage may be important in limiting heterologous gene expression in mammalian cells. As a result, modifying the codons of inserted genes prior to their insertion can make them reasonably compatible and more readily expressed.

Aptamers able to bind *Orthopoxviruses* have been developed (479).

German scientists reported that an infection in a colony of 80 monkeys in 2002 with 30 deaths was caused by a poxvirus related to cowpox virus (480). The monkeys had been exposed to rodents that showed evidence of prior poxvirus infection; rodents are known to be a reservoir for cowpox (480).

PCR reactions have been developed to distinguish among the various *Orthopoxviruses* (202, 481) and to diagnose cowpox in a human (203). The assay was 100 times more sensitive than traditional virological culture on Vero cells. German scientists also have developed a PCR assay followed by a DNA melting analysis for detection of *Orthopoxviruses* and of variola virus specifically (204, 205). A RAPD assay has been used to discriminate closely related cowpox viruses (482); however, PCR plus sequencing may be more specific.

A large percentage of cowpox infections of humans have been traced to cats (203, 240), which apparently contract the virus while hunting cowpox-carrying rodents.

A certain major histocompatibility complex type (HLA-A*201) has been associated with cytotoxic T cell responses to a variola virus epitope (24). This finding may be one factor, along with preprogrammed cytokine production profiles, that underlies differences among people or groups of people in susceptibility to the virus.

German scientists have found that a vaccinia virus gene product associates with host cell membranes, including those of the endoplasmic reticulum, in helping to determine the cytoplasmic sites of viral replication (263, 483).

Studies with a modified vaccinia virus Ankara, typically used to carry genes for immunization or medical treatments, were conducted to maximize efficient production of recombinant viruses (484). In other experiments with the modified vaccinia virus, a

method was developed to rapidly determine neutralizing antibodies in smallpox immunization efforts (485). Indeed, a modified vaccinia virus Ankara has been shown to protect mice from a respiratory challenge with virulent vaccinia virus (24); however, the applicability of that approach to humans and the strength and duration of protection remain to be determined. One paper dealt with the risks of vaccination in people with skin diseases (486).

Like scientists from many other countries, German scientists have used modified vaccinia viruses to deliver genes or to carry genes for antigens from pathogens for immunizations (487-492). Genetic requirements for *in vitro* propagation of this virus have been studied (491, 493-495).

Studies in chickens showed that vaccinia infection induced interferon-beta as well as interferon alpha and gamma (496).

German scientists have tried to develop anti-viral agents that might be effective against a poxvirus infection (497). Unfortunately many of the compounds tested – (i) cyclic and acyclic nucleoside analogues, (ii) a prodrug of cidofovir, (iii) humic acid type phenolic polymers, and (iv) pyrophosphate analogue – have been rather toxic.

It was found that the frequency of secondary transmissions of variola might be highest between three and six days after onset of fever (498). Such information could be used to improve models of smallpox spread and for public health planning.

United Kingdom

A camelpox protein and vaccinia proteins were found to modulate apoptosis of host cells; they interact with Bcl2 (432, 499, 500). Such viruses also have the ability to modulate host cell actin filaments, which enhances cell-to-cell spread of the virus (501). Moreover, a vaccinia virus virulence factor, N1, has a Bcl-2-like anti-apoptosis action (502).

Sequencing and analysis of camelpox virus led to the discovery that the camelpox virus is most closely related to variola virus, the causative agent of smallpox (90). Both produce the same kind of small white pocks on the chorioallantoic membranes of fertilized hens' eggs and have a similar ceiling temperature for the growth of the pocks. Both viruses are unable to grow in rabbit skin. Comparisons indicate that (i) the genome sequences of the two viruses are closer than either is to any other virus, (ii) the arrangements of the open reading frames (protein coding sequences) are most similar to each other, (iii) the protein sequences themselves are most closely related to each other, and (iv) the nature and structure of the terminal repeats are most similar between variola and camelpox viruses (90). Nonetheless, the number of genetic alterations that would have to be made in camelpox virus to convert it into a serious human pathogen is uncertain.

The camelopox virus encodes a schlafen-like protein that has a mixed affect on virulence (91). This gene is disrupted in variola virus, which could possibly contribute to increased

virulence. Disruption of the camelpox virus gene could possibly increase the virulence of that virus.

British scientists engineered vaccinia viruses to carry Type III (lambda) interferons with no increase in virulence (503).

British scientists studied the immune cells entering the site of a vaccination in a mouse model (504). Viral strains lacking the kelch protein A55 had larger than normal skin lesions (505). They also found that protein B14 is a vaccinia virulence factor (506). Other virulence factors also were studied (52-58). Manipulation of some of these could possibly increase virulence. Of note, inhibition of IL18 was found to increase virulence (56), which could point to various approaches to interfering with IL18 so as to increase virulence.

Vaccinia virus was found to encode an inhibitor of apoptosis, the result of which would be to allow the virus to replicate without host cells dying before viral progeny could be released to continue the infection (507).

Methods, including PCR assays, have been developed for detection of variola virus and *Orthopoxviruses* generally (206).

The variola complement control protein is more potent than the vaccinia complement control protein, which may contribute to the increased virulence of variola virus (272).

The *Orthopoxvirus* protein that binds interferon *gamma* is a dimer in vaccinia, cowpox, and camelpox viruses (273).

Human cowpox infections occur in Europe. Between two and four cases are reported from the UK each year. These infections are commonly contracted from domestic cats that acquire the virus while hunting virus-carrying small rodents (239).

It has been suggested that an allele in European populations may have been under selective pressure in the past because of smallpox (508).

The exit of mature *Orthopoxvirus* particles from host cells has been under consideration (509-511). In addition, viral effects in skin appear to be different from those in other organs (512, 513).

The *Orthopoxvirus* thymidine kinase has been studied in terms of association with other molecules and as a potential target for treatment (514).

Newer antiviral nucleosides are being developed as anti-poxvirus agents (515, 516). A DNA molecule containing immune-stimulatory CpG sequences also may assist in the therapy of *Orthopoxvirus* infections (124).

The adverse reactions of 200 vaccinated health care workers were recorded (517); however, this represents too small a number of people to provide meaningful population information. Modified vaccinia Ankara, a virus that does not replicate in humans, was studied as a possible smallpox vaccine (518). New tissue culture derived smallpox vaccines are being developed (519). British scientists also have been studying the potential use of DNA vaccines against smallpox in a mouse model (520). Such vaccines induce good interferon *gamma* responses but low antibody responses.

British scientists argue that retention of the B5 protein is critical for a smallpox vaccine because that immunogen is what is recognized by important antibodies (285).

Vaccinia immune globulin was tested in a mouse model of disease (521). It was found that the immune globulin might be useful for treating reactions to vaccination with vaccinia virus but would not have a high enough titer to treat smallpox infections.

British scientists have been using modified vaccinia viruses to deliver genes or to carry antigens for immunization against various infections, including malaria and HIV, and in cancer therapy (51, 522-555). They found that deletion of gene A41L increased vaccinia virus immunogenicity (556).

In addition, a vaccinia virus CC-chemokine inhibitor is being developed to treat atherosclerosis (557, 558).

Mathematical models of a potential smallpox outbreak have been developed (559). They emphasize the importance of contact tracing and quarantining; however, these measures are only effective when the outbreak is discovered very early in its evolution.

Ireland

Mechanisms used by vaccinia virus to interfere with host immune responses were studied (268-270, 275). These included actions on (i) NuclearFactor-*kappa*B, a transcription factor involved in global host immune regulation, (ii) IL10, (iii) TLRs, (iv) IL1, (v) IL18, and (vi) TNF and its receptor, with complex molecular interactions.

Iceland

Scientists studied the effect of the *Orthopoxvirus* complement-binding protein on atherosclerosis in mice (560).

Denmark

In a study done in Guinea-Bissau, Danish scientists found that prior vaccination with vaccinia virus was associated with improved mortality in adults for unknown reasons, but apparently was unrelated to poxvirus infections (561).

Vaccinia virus was found to stimulate dendritic cells, which may play a role in its use as a carrier for various immunogens (190).

Sweden

Swedish scientists, like those of other nations, have used vaccinia viruses as a vector to deliver antigens or genes (180). They also have developed detection methods for *Orthopoxviruses*.

Swedish scientists have studied complement-binding proteins, including the vaccinia virus complement binding protein. They found that the vaccinia virus complement-binding protein is quite stable and is composed of certain specific structural domains, which may provide the stability (276).

Cowpox infections occur in Sweden (243).

Norway

A modified vaccinia virus is being developed as a vector for immunization against infectious diseases and for cancer treatment (562).

Norwegian scientists tried to recombine several poxviruses, including cowpox virus (109). If done by an adversary, such experiments would be of some concern.

To better understand the details of vaccinia virus infection of human cells, scientists in Norway have analyzed effects of vaccinia infection of a human cell line (563).

Cowpox infections occur in Norway and Sweden (242, 567).

Finland

Human cowpox disease currently is contracted primarily from infected domestic cats, who in turn contract the virus from rodents, although dogs also may be implicated (245).

A vaccinia protein with anti-inflammatory properties has been used in graft treatment (564).

Netherlands

Vaccinia virus was used as a vector for tumor-associated antigens in cancer therapy (62) or in immunization against another infection (63, 64, 565, 566).

A generalized infection with cowpox virus was found in an animal in an animal park (237).

Experimental monkeys were infected via the trachea with lethal monkeypox virus. Those monkeys given an acyclic nucleoside phosphonate drug starting 24 hours after infection and continuing for 13 days responded better than monkeys vaccinated with the human vaccinia vaccine given 24 hours after infection (125).

Topoisomerases cut and rejoin one strand of a DNA duplex which allows removal of supercoiled regions of the DNA that can form during replication and/or transcription. The interaction between the vaccinia virus topoisomerase IB enzyme and DNA was studied (567).

Mathematical models of a smallpox outbreak suggested that ring (contact) vaccination can be successful if instituted rapidly (35).

Austria

A joint Austrian-US effort to produce quantities of smallpox vaccine involved cloning of the old Dryvax vaccinia virus strains and growing up batches in tissue culture (568). Some of the clones were unacceptably virulent in animals. One less virulent strain, found to be as immunogenic as the original Dryvax, was selected for use as the new vaccine and grown in MRC-5 human diploid cells (568). This vaccine induced both cell-mediated immune responses and neutralizing antibody. However, it turned out to be as reactogenic as Dryvax, which is thought by many to be unacceptable for routine immunizations, even if it might be used in a dire emergency. They have also developed a method for the generation of replication – defective vaccinia viruses that could be used as vectors (569). One such modified vaccinia was found to produce long-lasting immunity in mice with the expectation of fewer adverse reactions in humans (61); nonetheless, studies from other countries suggest that replication-defective vaccinia viruses do not produce persistant immunity in humans as do viruses that replicate.

Efforts at filtration of the virus during vaccine preparation were encouraging (570).

Austrian scientists have reviewed the use of vaccinia virus as a vector for a variety of genes (60, 571).

Belgium

More than 50 years ago, thiosemicarbazones were found to have anti-viral activity, and one of those, methisazone, was investigated in the prophylaxis of smallpox (131). However, with the effective use of vaccinia as a smallpox vaccine, such studies of thiosemicarbazones were abandoned (131).

Cidofovir, used in the therapy of AIDS, also has good activity against *Orthopoxviruses* (572, 573). One newer drug type with activity against *Orthopoxviruses* is an acyclic nucleoside phosphonate [a pyrimidine with a C-2 amino group and a C-6-2 (phosphonomethoxy)ethoxy group or with another phosphonate group] (129). The base consists of a pyrimidine with an amino group at C-2. The inhibition was similar to that of cidofovir, to which some poxviruses are becoming resistant (129). Other nucleoside and nucleotide analogues also have been found to have antiviral activity and could possibly be developed for anti-smallpox purposes (127, 574, 575). Other drugs might target viral enzymes, such as DNA polymerase (131).

Belgian scientists have been interested in the use of poxviruses as vectors for other antigens (576).

A joint British, Belgian and US group found that a Yaba-like disease virus produced an IL10-like protein that could modulate host immune responses after a poxvirus infection (577). Although many cytokine analogues worsen poxvirus diseases, this protein may not. Additional work will be needed to determine its role in naturally occurring poxvirus infections.

Belgian workers analyzed thymidylate kinase from vaccinia virus with the aim of developing new anti-*Orthopoxvirus* drugs directed at that enzyme (126).

Nomadic herders have developed their own vocabulary for dealing with camel diseases, including camelpox (578). Native Taureg populations of Niger can identify *erk eshik* (camelpox) and distinguish it from other disorders of camels.

Egypt

Egyptian scientists reported an outbreak of buffalopox on a private farm containing 216 buffalo (579). The attack rate was 8%. Disease severity varied from mild to fatal.

A vaccinia virus encoding T7 polymerase was used as an expression vector system (580).

Saudi Arabia

Sheep and goats were immunized with a sheeppox live vaccine from Romania to counter an outbreak of capripox (581).

South Africa

Modified vaccinia virus was used by South African scientists as a vector for rabies antigens (582). A vaccinia virus immunosuppressive protein (the complement binding protein) has been studied for its ability to prolong graft survival after transplantation (583). The protein also causes improvement after experimental brain trauma (584) or spinal cord trauma (585). Humanizing the gene encoding that protein makes the engineered protein 100 times more potent (586). South African scientists also have found a potent virulence factor of vaccinia virus that enhances viral growth in the brain (587).

A joint Gambian, Senegal, and British study used the vaccinia virus as a vector for tuberculosis antigens (588). Gambian workers are using vaccinia for carrying malaria antigens for purposes of immunization (589, 590). Studies from Kenya use vaccinia for both malaria and HIV antigens (181).

An Ethiopian study assessed the economics of camels and the diseases affecting them (591).

Cuba

Cuban scientists have used vaccinia and fowlpox viruses in vaccination strategies against other diseases, including HIV (182, 183, 592).

Mexico

Mexico has developed a comprehensive national preparedness plan to be used in the event of a bioterrorism event with smallpox (593).

Mexican scientists have used vaccinia virus as a vector for other antigens (184, 594, 595).

Brazil

Faced with naturally occurring outbreaks of *Orthopoxviruses* that infect farm animals and people (195, 250-256, 596-601), Brazil has performed good work (i) on characterizing the viruses as vaccinia virus variants – many now naturally occurring throughout the country (598, 600, 601, 603, 604), (ii) in developing detection methods (250, 251, 255, 256, 600, 601, 605-607), and (iii) in studying aspects of the *Orthopoxvirus* replicative cycle within mammalian host cells (608-617). Brazilian scientists also have used vaccinia virus as a vector for carrying other antigens (185-187).

The genomes of vaccinia and related *Orthopoxviruses* contain genes that encode proteins that trick the host immune system (i) in a non-specific manner, or (ii) by their homology with host soluble factors or host receptors for such factors (598, 599). Among the latter

type are the IL1 beta inhibitor, a serine protease inhibitor that interferes with the enzyme IL1 convertase, and cysteine aspases (caspases) involved in apoptosis (598) as well as a protein that binds to interferon-1 and thereby serves as an inhibitor of interferon actions (599). These processes were studied in recombinant vaccinia viruses (598). In addition, the virus uses the early growth response gene (egr-1) to facilitate replication (616).

Outbreaks of vaccinia virus were found to affect both cattle and people in several regions of Brazil (195, 596, 597, 599, 600). Affected people often had a poxvirus rash (on the face and arms especially), lymphadenopathy, and fever. At least six strains of *Orthopoxviruses* have been isolated around Brazil (599, 604, 618); sometimes more than one strain was found in a given outbreak (195). Based upon analyses of thymidine kinase, vaccinia growth factor (VGF), hemagglutinin (HA), and acidophilic inclusion body genes, these appear to be vaccinia viruses or vaccinia-like viruses from various smallpox vaccination regimens, but which now have become established in natural habitats (598, 600, 601, 604). In addition, unusual variability has been observed (619).

Studies show that vaccinia virus induces extracellular signal-regulated kinases (ERKs) early after infection (609, 612, 614, 620, 621). This induction appears to play a critical role in allowing viral replication within the host cell (612). Mitogen-activated protein kinase (MAPK) signaling pathways MAPK JNK and p38 are activated in host cells late in infection (609, 614, 620). The vaccinia growth factor (VGF) is important in this latter activation (620, 622). Some similar results were found for cowpox virus; however, cowpox and vaccinia viruses recruit different pathways for JNK activation (610, 611, 621, 622).

The early response to growth gene Egr-1 of vaccinia virus appears to encode an important transcription factor impinged upon by signals involved in cell cycle progression, differentiation, and apoptosis (611, 615). Such factors as hormones, neurotransmitters, and growth and differentiation factors stimulate Egr-1 (611).

Scientists in Brazil studied the vaccinia virus F11L protein, which is expressed early in infection but for which no function had been described (608, 613). This gene product was found to interact with the G2 post-replicative transcription elongation factor. Additional studies show that F11L shifts the ratio of extracellular enveloped viral particles and cell-associated enveloped viral particles, which can increase virulence (608).

Lambda interferons were found to have little effect on vaccinia virus (623).

A study of FK506 suggests that the drug may have anti-*Orthopoxvirus* activity targeting the stage of viral DNA replication (624).

Argentina

Like many countries, Argentina has studied antibody titers to vaccinia virus used in prior vaccination of people against smallpox. A good inverse correlation was found between antibodies and time since last vaccination (625). However, virus neutralizing antibodies were less frequent than antibodies measured by an immunoassay, suggesting that much of the population is not protected against a potential recurrence of smallpox.

Modified vaccinia virus Ankara, which does not replicate in recipient mammalian cells, is being developed for use as a carrier for other genes (188, 189).

In light of the potential for a recurrence of smallpox, scientists studied an old batch of smallpox vaccine called the Malbran strain, produced between 1945 and 1949. Live virus was grown and found to protect mice from cowpox virus challenge (626). The strain was related to the Lister strain. This work shows that a 60-year-old *Orthopoxvirus* is still able to be grown up and used.

Canada

Tests were conducted to verify the potency of smallpox vaccine stocks (627). The chorioallantoic membrane test and Vero assays for vaccinia virus strains were re-established for this work. Also, in light of the concerns about bovine spongiform encephalopathy (known as Mad Cow Disease) in North America, vaccine production will proceed without the use of animal proteins in the culture media (132).

A family of poxvirus virulence proteins as studied (628). These proteins were involved in localization of daughter virus production within the cytoplasm of an infected cell and with a role in apoptosis (628).

The *Orthopoxvirus* glutaredoxin gene product was studied by X-ray crystallography (629, 630). In other studies, *Chordopox* virion structure was analyzed (631).

Scientists in Canada, like those in many other countries, have used vaccinia virus as a vector for antigens in vaccine development for other (non-*Orthopoxvirus*) infectious diseases or for gene transfers in the therapy of cancer and other medical conditions (84-88, 632-637).

Infection of human host cells by vaccinia virus causes down-regulation of MHC I molecules on those host cells, which predisposes them to elimination by natural killer (NK) cells (638). The virus also interferes with NK cell functions that might eliminate vaccinia virus. A vaccinia virus F1L protein allows virus-carrying macrophages to survive and viral replication to proceed (639, 640).

A Canadian group developed bioinformatics methods to identify functional genes in *Orthopoxviruses* by identifying purine skewing to predict their A-T rich genes (641). This group also analyzed the G5R protein of vaccinia virus, explored its evolutionary relationships, and found similarities to a human protein (642).

Canadian scientists sequenced a rabbitpox virus genome and compared it with vaccinia virus sequences (196). The work confirms that rabbitpox virus is closely related to vaccinia virus but indicates that rabbitpox virus did not directly evolve from any sequenced vaccinia virus strain (196).

Studies of vaccinia virus associated with a vaccine complication revealed that (i) Dryvax vaccine is not clonal but contains several vaccinia variants, and (ii) the viral lesions in the patient with progressive vaccinia disease appeared to be clonal (643).

In recent years, it has been found that RNA interference (RNAi) can allow mammalian cells to resist viral infection. The vaccinia virus EL3 protein can inhibit RNAi – perhaps via inhibition of interferon-induced protein kinase R (PKR) – thereby reducing this anti-viral effect (644).

Orthopoxviruses can recombine within a host cell. Canadian scientists found that the vaccinia virus DNA polymerase had certain properties that allowed such recombination (112, 645).

A Canadian cat was found to be infected by racoonpox virus (646).

The antiviral drug cidofovir was found to inhibit the vaccinia virus DNA polymerase, a mechanism that may be critical to the anti-poxvirus effects of the drug (647). Canadian scientists also have been studying the mechanism of action of another anti-viral, ribavirin (648).

Canadian scientists studied the phenomenon of heterologous reactivation (106). This is a process whereby a cell infected with one poxvirus can produce infectious virus from the pure DNA of a second poxvirus. Such a method could allow resurrection of a no longer extant poxvirus from its known DNA sequence after synthesis of the DNA itself.

Discussion of Treatment

Over the years, the effectiveness of vaccination against smallpox with vaccinia virus reduced the impetus to develop drugs effective against the virus for use either in prophylaxis or in therapy. However, with the eradication of smallpox as a naturally occurring disease and suspension of routine public health vaccination programs, it was recognized that vaccinia virus caused unacceptable adverse side effects in too many recipients to justify its use in large populations against a disease that no longer existed in nature. However, in a desire to be prepared for a possible terrorist attack with variola virus, many countries have tried to develop either less toxic vaccines or new drugs that

might prevent or treat smallpox or a related disease. Such developmental work has covered (i) development of vaccines with fewer adverse events, and (ii) testing of known drugs and new compounds for potential use as anti-*Orthopoxvirus* agents. This type of work is ongoing in several countries and has yielded some promising results.

Vaccination with a standard vaccinia virus preparation causes a mild viral disease and stimulates an immune response that protects against other *Orthopoxviruses*. Many people with atopic dermatitis (a large group, as ~10% of Americans have had eczema) do not produce two antimicrobial peptides: cathelicidinia and beta-detensina in amounts sufficient to prevent serious systemic vaccinia disease (121). This condition is called eczema vaccinatum, which is most often contracted from a family member or other contact who has been vaccinated. Australian scientists have made similar observations (649).

A study of FK506 suggests that the drug may have anti-*Orthopoxvirus* activity, targeting the stage of viral DNA replication (624).

A US team produced monoclonal antibodies to a glycoprotein (A33 glycoprotein) of vaccinia virus and to another antigen (B5 protein) of vaccinia virus (650, 651). The monoclonals also bound to the homologous variola virus antigenic determinants and inhibited the spread of variola *in vitro*. Such monoclonal antibody reagents might be suitable for use in treatment of complications of vaccinations with vaccinia virus or against the spread of smallpox, should there be a new outbreak of that disease, or against monkeypox.

Live vaccines for smallpox require refrigerated transport and storage (29), leading to investigations into alternative approaches. Peptide vaccines have been considered; they may not provide long-term immunity and might not induce immunity in people with certain MHC types (29). In addition, toxic adjuvants may be required to induce an adequate immune response with peptide antigens. A lipid-core-peptide system that incorporates a lipid adjuvant, carrier, and peptide antigen may overcome these drawbacks (28, 29). Moreover, particles with both immunological adjuvant and carrier functions activate host dendritic cells, which is a critical feature for effective immunogens in vaccines (28).

Russian scientists have been evaluating an oral form of live vaccinia virus as a human vaccine for decades. This oral vaccine showed far fewer adverse reactions than the standard skin scarification method and provided protection of people from smallpox in Ethiopia in 1972-1973 (27). Russian scientists also have developed a DNA vaccine against variola virus, so far tested in mice (26).

More than 50 years ago, thiosemicarbazones were found to have anti-smallpox activity (131). However, with the effective use of vaccinia as a smallpox vaccine, studies of thiosemicarbazones were abandoned (131). Cidofovir has good activity against orthopoxviruses. Other drugs might target (i) cellular enzymes, such as IMP dehydrogenase, SAH hydrolase, OMP decarboxylase, and CTP synthetase, and (ii) viral

enzymes, such as DNA polymerase (131). One newer drug type with activity against *Orthopoxviruses* is an acyclic nucleoside phosphonate [a pyrimidine with a C-2 amino group and a C-6-2 (phosphonomethoxy)ethoxy group or with another phosphonate group] (129). The base consists of a pyrimidine with an amino group at C-2. The inhibition was similar to that of cidofovir, to which some *Orthopoxviruses* are becoming resistant (129, 130). Other nucleoside and nucleotide analogues also have been found to have antiviral activity and could possibly be developed for anti-smallpox purposes (127, 128).

Ribavirin appears to have some activity against *Orthopoxviruses*. Combinations of anti-viral drugs may be useful (342).

In a collaborative study between Czech and US scientists, it was found that vaccinia virus strains made resistant to cidofovir during serial passage in Vero cells in culture had cross-resistance to compounds related to cidofovir but not to unrelated nucleosides (130). This result raises the issue of potential purposeful development of a virulent *Orthopoxvirus* pathogenic for humans that might be resistant to the most promising anti-smallpox drugs currently under serious study.

In searching for anti-variola drugs, it was found that delivery systems may be critical because of the poor *in vivo* conversion of nucleoside analogue drugs to their active triphosphate nucleotide forms (467). In an attempt to overcome this problem, the pro-drugs were encapsulated into a poly(iso-butylcyanoacrylate) aqueous core nanocapsule. However, this did not work as well for these mononucleotides as had been found for oligonucleotides (467). Additional work on formulation will be required.

French and Belgian workers analyzed thymidylate kinase from vaccinia virus with the aim of developing new anti-*Orthopoxvirus* drugs directed at that enzyme (126).

Lethal intra-tracheal infection of monkeys with monkeypox virus was found to respond better to an acyclic nucleoside phosphonate drug started 24 hours after infection and given for a 13-day period, rather than to vaccination with the human vaccine given 24 hours after infection (125).

Unfortunately, some drugs with activity against monkeypox virus and other *Orthopoxviruses* may have limited activity against variola virus (652).

A DNA molecule containing immune-stimulatory CpG sequences also may assist in the therapy of *Orthopoxvirus* infections (124).

Non-replicating vaccinia viruses were studied for utility as potential smallpox vaccines in a mouse cowpox model (25). These conferred less long-term immunity than classical replicating vaccinia vaccine strains. A second generation vaccine would consist of live virus grown in tissue culture (such as human fetal lung fibroblasts) under aseptic conditions (122, 466). Second generation vaccines eliminate concerns about contamination of calf-lymph derived vaccinia with such things as microbes, prions that

might induce Mad Cow Disease, or bovine antigens in the preparation that could cause undesirable immune responses.

A third-generation vaccine would have much enhanced safety and yet retain an ability to induce a long-lasting immune response (466). Although a modified vaccinia virus Ankara has been shown to protect mice from a respiratory challenge with virulent vaccinia virus (24), the applicability of that approach to humans and the strength and duration of protection remain to be demonstrated.

A strain of vaccinia virus that had been used as a smallpox vaccine was found by Japanese scientists to become more virulent after growth in culture due to a mutation in a single gene (283). That gene could be eliminated without loss of protective immunity (283). The resulting strain was as protective as the US Dryvax strain and yet much less virulent. Additional work on the vaccine strain lacking the complicating B5R gene has produced what appears to be a much safer vaccine, one that might be sufficiently devoid of serious adverse effects to be considered for large population groups (21-23). This vaccine appears to protect early in infection by inducing cell-mediated immunity more than antibodies (284). However, British scientists argue that B5 is critical for an immunogen because it is what is recognized by important antibodies (285). Thus, although progress is being made on both new vaccines and new drugs for poxvirus infections, work continues in the search for better pharmaceuticals.

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