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ACAM2000 clonal Vero cell culture vaccinia virus (New York City Board of Health strain) — a second-generation smallpox vaccine for biological defense

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Summary The threat of smallpox as a biological weapon has spurred efforts to create stockpiles of vaccine for emergency preparedness. In lieu of preparing vaccine in animal skin (the original method), we cloned vaccinia virus (New York City Board of Health strain, Dryvax®) by plaque purification and amplified the clone in cell culture. The overarching goal was to produce a modern vaccine that was equivalent to the currently licensed Dryvax® in its preclinical and clinical properties, and could thus reliably protect humans against smallpox. A variety of clones were evaluated, and many were unacceptably virulent in animal models. One clonal virus (ACAM1000) was selected and produced at clinical grade in MRC-5 human diploid cells. ACAM1000 was comparable to Dryvax® in immunogenicity and protective activity but was less neurovirulent for mice and nonhuman primates. To meet requirements for large quantities of vaccine after the events of September 11th 2001, the ACAM1000 master virus seed was used to prepare vaccine (designated ACAM2000) at large scale in Vero cells under serum-free conditions. The genomes of ACAM1000 and ACAM2000 had identical nucleotide sequences, and the vaccines had comparable biological phenotypes. ACAM1000 and ACAM2000 were evaluated in three Phase 1 clinical trials. The vaccines produced major cutaneous reactions and evoked neutralizing antibody and cell-mediated immune responses in the vast majority of subjects and had a reactogenicity profile similar to that of Dryvax®.

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Background and Rationale

In 1980, the World health Organization (WHO) declared that smallpox had been eradicated from the face of the Earth. This was achieved by an intensified international program of surveillance and vaccination. In 1980, there were uncertainties about the potential reappearance of the disease, and preparations were made for a large reserve stockpile of vaccine (200 million doses) to be held in Geneva and New Delhi. At the time, there was minimal concern about smallpox as a bioweapon. By 1984 only two controlled repositories of variola strains remained (in Moscow and Atlanta), making attribution clear in case of a biological attack. Moreover the high prevalence of vaccine immunity was thought to be a disincentive to the use of variola as a weapon. By 1986, in the absence of any recrudescence of smallpox, WHO reduced its vaccine repository to approximately 500,000 doses. Moreover, smallpox vaccine manufacturing had virtually ceased worldwide in the absence of vaccination programs and any commercial incentive for production.

Smallpox had virtually disappeared from the United States and Europe (except for occasional imported cases) a decade before global eradication. With the diminishing threat of smallpox and increased focus on adverse events,[1,2] vaccination in the United States was discontinued in 1972 for the general public and in 1989 for military personnel. With each passing year, the population thus became increasingly vulnerable to the virus. In the mid 1990s, the United States Government became alarmed about the threat of smallpox as an agent of biological terrorism.[3] The concern stemmed from revelations by defectors from the former Soviet Union, especially Kanatjan Alibekov (Ken Alibek).[4] He disclosed the existence of a sophisticated program beginning shortly after World War II and culminating in the 1980s with large-scale production of a virulent variola strain, and positioning of strategic long-range bombers and SS-18 multiple warhead intercontinental missiles armed with munitions containing the virus. Although the Soviet biological program was officially renounced by President Yeltzin in 1991, destruction of these weapons has never been verified, and new concerns have arisen about proliferation of smallpox stocks in other countries having adversarial relations with the West. Between Alibek's defection in 1992 and 1998, United States policy was shaped accordingly. In 1998, President Clinton announced a program to provide countermeasures, including a stockpile of vaccines. Only 15 million doses of smallpox vaccine remained in the US, and these doses had been manufactured 20 years earlier.

In July, 1999, the Centers for Disease Control and Prevention (CDC) solicited expressions of interest from industry in manufacturing a 40 million dose stockpile of a modern cell culture smallpox vaccine, and in September 2000, a contract for vaccine production was awarded to Acambis. After the events of September 11, 2001, the terms of the original contract were modified to increase deliverable doses from 40 to 54 million. In November 2001, Acambis successfully competed for and was awarded a second contract for an additional 155 million doses of vaccine, bringing the total requirement to 209 million doses. Both CDC contracts specified that the new vaccine be delivered in the shortest possible timeframe and that it be licensed by the US Food & Drug Administration (FDA) based on successful clinical trials. The Government's intention was to have "1 dose available for every citizen". The balance of doses between the Acambis contracts and the US population was to be drawn from the original stockpile of 15 million doses of licensed vaccine [Dryvax® (New York City Board of Health strain), Wyeth Laboratories], which (based on new clinical trial data[5,6]) could be diluted 1:5 for administration to humans in an emergency. In order to deliver such a large quantity of vaccine, Acambis partnered with Baxter BioScience, which had large-scale bioreactor capacity at its facilities near Vienna, Austria.

Development of a new cell culture vaccine

First generation smallpox vaccines are manufactured from lymph collected from the skin of live animals (most often calves or sheep) that have been scarified with vaccinia virus. This method of manufacture came into widespread use in the second half of the 19th Century. Although first generation vaccines are still made in the Netherlands, Russia and China, this method of manufacture is highly undesirable, particularly with respect to control of adventitious viruses and in light of advances in technology. Since vaccinia virus grows to high titers in a variety of cell culture systems acceptable for use in manufacturing human vaccines, the obvious objective was to manufacture the vaccine in an approved cell bank, preferably in continuous cells that had been controlled for adventitious agents and tumorgenicity. At the time the project began, there had been several successful historical attempts to produce cell culture smallpox vaccines, including viruses propagated in primary rabbit kidney cells[7,8] and in continuous MRC-5 human diploid lung fibroblast cells.[9]

A critical decision at initiation of the project was whether or not to establish a biological clone or to

simply pass the existing licensed vaccine in cell culture without cloning. The benefits of cloning appeared to outweigh the recognized risk that a clonal virus population may differ biologically from the 'genetic swarm' represented by the animal-skin vaccine.[10] Because it would not be possible to conduct field tests for efficacy, the new vaccine would need to match the licensed vaccine (Dryvax®) as closely as possible in preclinical tests for safety, immunogenicity, and protective activity and in clinical trials for safety and immunogenicity.

The initial development of a suitable vaccine candidate (designated ACAM1000) was recently described by Weltzin et al.[10] They isolated and characterized multiple clonal viruses and also passed the uncloned virus population in MRC-5 cells. Cloning or passage commenced by pooling of ten 100-dose vials from each of 3 different production lots of Dryvax®. Six clones were isolated by 3 sequential plaque-purifications at terminal dilution and amplified to produce virus stocks at MRC-5 passage (P) 5. The uncloned virus was produced by 3 passages in MRC-5 cells at a multiplicity of infection (MOI) of 0.001 plaque-forming units (pfu)/cell.

Although the clones were derived by plaque picking at terminal dilution and should represent the majority virus population in Dryvax®, significant differences among them were observed.[10] As noted below, Clones 1, 3, and 5 and the uncloned virus had virulence properties that were unacceptable for consideration as vaccine candidates. The results with the uncloned virus demonstrated that it would have been undesirable to manufacture by simply passing the original calf-skin virus in cell culture.

Preclinical safety in animal models

To determine if the viruses differed from Dryvax® in their ability to cause pock lesions in skin, the clonal and uncloned vaccine candidates were inoculated by the intradermal route in rabbits. Rabbits were inoculated in the paravertebral area with tenfold dilutions of a candidate virus and along the opposite side with graded doses of Dryvax®. On Day 8, the diameter of erythema and of the central lesion (ulcer) were measured. Dryvax® produced a maximum erythema of 20 mm and a maximum central lesion diameter of 3 mm. Lesion size was greater than Dryvax® for Clones 1, 3 and 5 and for the polyclonal virus, whereas Clones 2, 4, and 6 produced either no central lesions, or lesions that were similar to Dryvax®.

It has long been known that vaccinia strains differ with respect to neurovirulence in infant mice. The 6 clonal and 1 uncloned vaccine candidates were therefore compared to Dryvax® by intracerebral (IC) inoculation of suckling mice with $\log_{10}$ dilutions of virus. The same four viruses that had exhibited excessive virulence in rabbit skin (Clones 1, 3, and 5, and the polyclonal virus) were significantly more neurovirulent than Dryvax® ($p < 0.05$, Kaplan–Meier survival distribution, log rank test), whereas Clones 2, 4, and 6 were similar to Dryvax® or less virulent. The relative neurovirulence of the clonal viruses expressed as the median and 90% lethal dose is shown in Table 1. The more virulent viruses also replicated to higher titer in mouse brain.[10] In these initial experiments Clone 2 did not appear to be attenuated with respect to neurovirulence, but subsequent studies with larger numbers of animals showed significantly higher survival distribution compared to Dryvax® (Figure 1A). To confirm the attenuated phenotype of Clone 2 virus, two groups of 6 young adult rhesus monkeys were then inoculated by the intrathalamic route with 7 $\log_{10}$ pfu of ACAM1000 or Dryvax®. Three (50%) monkeys inoculated with Dryvax® but none of the animals inoculated with ACAM1000 developed severe neurological illness. At necropsy, the animals with fatal illness had severe non-purulent meningitis (Table 2).

Table 1 50% and 90% Lethal Doses (LD) of Dryvax® and vaccine candidates and average survival times (days) at a dose of 2.3 $\log_{10}$ plaque-forming units (pfu) inoculated by the intracerebral route.

Experiment	Virus	LD_{50}/0.02 mL	LD_{90}/0.02 mL	Ave survival time
1	Dryvax®	1.6	3.0	9.6
	Polyclonal	<1.3	1.9	6.1*
	Clone 1	<2.3	<2.3	4.2*
2	Dryvax®	<1.3	2.1	6.7
	Clone 2 (ACAM1000)	1.5	2.2	6.9
	Clone 3	<1.3	<1.3	5.0*
	Clone 4	2.6	3.2	14.4*
3	Dryvax®	2.2	4.0	7.9
	Clone 5	<1.3	<1.3	4.1*
	Clone 6	1.6	2.5	7.8

* Significantly different from Dryvax ($p < 0.05$, Kaplan–Meier survival distribution, log rank test).

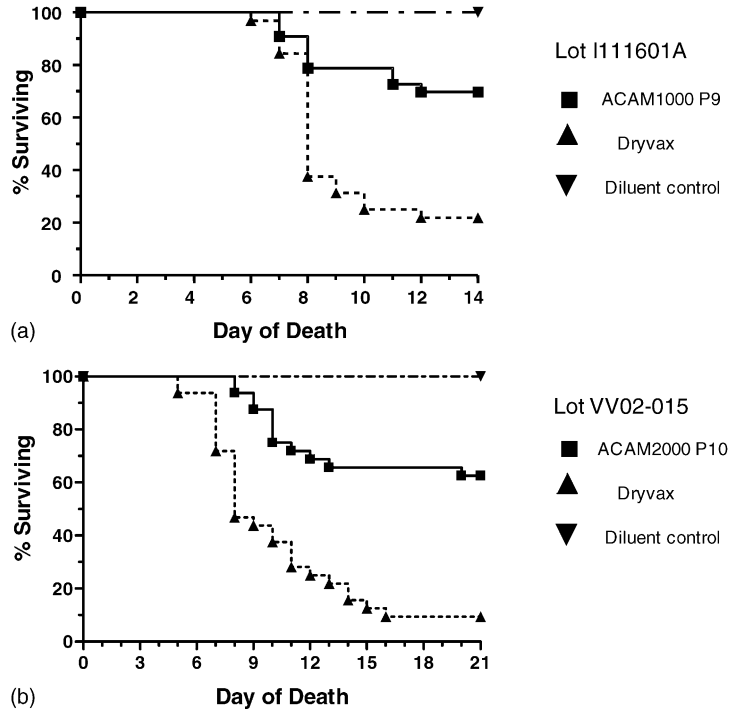

Figure 1 A and B Survival distributions of ACAM1000 and ACAM2000, respectively, compared to negative (diluent) control and Dryvax®. Groups of 32 suckling mice (4 litters of 8 mice) were inoculated by the intracerebral route with 2.3 $\log_{10}$ Vero cell plaque forming units of each virus in 20 μL or with diluent. The survival distributions of ACAM vaccines and Dryvax® are significantly different ($p < 0.05$, long rank test).

Table 2 Mortality ratio and average clinical scores, monkeys inoculated by the intrathalamic route with ACAM1000 or Dryvax® and necropsied on Day 16 after infection.

Study	Group (test article)	Dose $\log_{10}$ pfu	No. monkeys	No. (%) ill[a]	No. (%) dead	No. (%) severe histopathology[b]
1	ACAM1000	6.0	2	0 (0)	0 (0)	0 (0)
		7.0	2	0 (0)	0 (0)	0 (0)
		8.0	2	0 (0)	0 (0)	0 (0)
2	ACAM1000	7.1	6	0 (0)	0 (0)	0 (0)
	Dryvax®	7.7	6	3 (50)	3 (50)	3 (50)

[a] A clinical scoring system was used to assign each animal a daily numerical score. Signs of encephalitis, such as paresis, incoordination, lethargy, tremors or spasticity were assigned numerical values for severity by the following grading method: 0 = No clinical signs of encephalitis; 1 = Rough coat, not eating; 2 = High pitched voice, inactive, slow moving; 3 = Shaky movements, tremors, in coordination, limb weakness; 4 = Inability to stand, limb paralysis, moribund or dead. Animals euthanized due to illness deemed too severe to permit further observation under humane conditions were given a score of 4 on the day of euthanasia and a score of 4 from the day after euthanasia through Day 16. Animals that died were given a score of 4 from the day of death to Day 16. The mean clinical score for each monkey was the average of the animal's daily score; since animals were evaluated twice daily, the higher daily score was used. The clinical score for a group was the mean of the individual animal clinical scores. In Study 2, one Dryvax®-treated animal was found dead on Day 4, and two additional Dryvax®-treated animals underwent unscheduled euthanasia due to declining health status on Day 6. Clinical signs in these animals included decreased activity levels (observed as low food consumption, decreased activity, hunched appearance, and/or lethargic), tremors, seizures and convulsions, and loss of coordination. The 3 monkeys that developed clinical signs had individual mean scores of 3.1, 3.3 and 3.4. The group mean score for this treatment group was 1.8. None of the ACAM1000 monkeys showed signs of illness, and the group mean score was 0.4.

[b] Brain sections examined included cerebral cortex, basal ganglia (two levels), thalamus and midbrain, upper medulla and cerebellum, lower medulla and cerebellum, low medulla, and the cervical and lumbar spinal cord enlargements. Evidence of severe meningitis was noted histologically in the 3 early death Dryvax®-treated animals in Study 2. Mild signs of residual meningitis, such as edema and small areas of cellular infiltration (mostly by lymphoid and monocytic cells), were also noted in the remaining three Group 1 animals and 5/6 Group 2 animals. There was no degeneration or necrosis of neurons, and there were no neuronophagic foci. No accumulations of bacteria were found in brain sections from animals showing the most severe signs of meningitis, and there were no signs of demyelination. The characteristics of the neuropathological lesions are consistent with previous studies of vaccinia virus, which causes nonpurulent meningitis and brain edema in monkeys by, Morita et al.[24]

ACAM1000 was thus less neurovirulent than Dryvax® in both mice and nonhuman primates.

Clone 2 (renamed ACAM1000) was selected as the candidate for further development, based on its similarity to Dryvax® in pock formation in rabbit skin but its lower neurovirulence in mice and monkeys. In addition, Weltzin et al.[10] reported that ACAM1000 was similar to Dryvax® in plaque morphology and Hind*III* restriction analysis.

Preclinical studies of immunogenicity and protective activity

The immunogenicity of ACAM1000 was studied in mice and nonhuman primates. Protective activity was determined in mice challenged with cowpox and vaccinia Western Reserve (WR) viruses by the intranasal (IN) route and with ectromelia in mice challenged by the respiratory route with small particle aerosols. These studies, some of which have been published,[10] confirmed that ACAM1000 was similar to Dryvax® in its ability to induce pock lesions in mouse or monkey skin, neutralizing antibodies, and T cell responses, and to protect animals against lethal challenge. These studies are summarized in Table 3.

Based on these data, a pilot lot of ACAM1000 was manufactured at clinical grade. The virus was propagated in MRC-5 cells grown in cell factories, harvested from the disrupted cells, and purified by ultrafiltration and diafiltration. A Phase 1 randomized, double-blind clinical trial was conducted in 60 healthy adults aged 18–29 years who had not previously been vaccinated.[10] A second open-label study was subsequently conducted in 70 subjects. The results of these Phase 1 trials indicated that ACAM1000 was well tolerated and immunogenic (see Clinical Trials).

Development of ACAM2000

The requirement to rapidly produce 209 million doses of vaccine for the US national stockpile created a logistical problem for Acambis, which was still in the process of acquiring large-scale bioreactors for ACAM1000 viral production in MRC-5 cells. To resolve this problem, Acambis teamed with Baxter BioScience, which had large-scale production capabilities in its facilities near Vienna, Austria. The Baxter process utilized a different cell line [African green monkey kidney (Vero)] grown on microcarriers in a 1200 L bioreactor. It was necessary therefore to pass the ACAM1000 master virus seed in Vero cells to make a new production virus seed stock at P8 (Figure 2). To accommodate the scale of manufacture, an amplification passage (P9) needed to be introduced to provide sufficient virus to infect cells in the large bioreactor. Since both the cell substrate and passage level of the vaccine would change, molecular, preclinical and clinical bridging studies would be required to confirm that the new Vero cell vaccine (named ACAM2000) at P10 was an acceptable vaccine candidate.

Method of manufacture and control tests for adventitious agents

ACAM2000 was manufactured by infecting Vero cells grown on microcarriers under serum-free conditions with the P9 production virus inoculum at an MOI of 0.01–0.2. After allowing time for virus replication (approximately 3 days), the cells are harvested from the microcarriers and the intracellular virus is released by mechanical disruption. Cell debris is removed by large-pore depth filtration. Host cell DNA is digested with endonuclease (Benzonase®). Virus particles are subsequently purified and concentrated by tangential-flow filtration, followed by diafiltration. The resulting concentrated bulk vaccine is formulated by dilution with a buffer containing stabilizers to a final potency of $1.0–5.0 \times 10^8$ pfu/mL, filled into vials containing 0.3 mL (100 nominal doses), and lyophilized. For use, the lyophilized vaccine is reconstituted by addition of diluent [glycerol (50% v/v)-phenol (0.25% v/v) in water for injection]. Vaccine and diluent were labeled and packaged in kits containing 50 vials (5000 doses) and supplied with syringes for diluent transfer for reconstituting vaccine, and individually wrapped sterile bifurcated needles for administering vaccine to patients.

Because the original Dryvax® vaccine from which ACAM1000 and ACAM2000 was derived had an uncertain provenance and passage history in animal tissue, it was important to subject the seed viruses and vaccine lots to scrutiny for adventitious agents, including bacteria, fungi, mycoplasma and viruses. The approach that was taken to exclude the presence of detectable adventitious viruses went beyond compendial requirements (Table 4). To perform tests for adventitious viruses in cell cultures and animals, it was necessary to neutralize the vaccinia virus with polyclonal and monoclonal antibodies. In addition, seed viruses and vaccine produced from each bioreactor run were tested for neurovirulence in suckling mice, using Dryvax® as a comparator. Residual Vero cell DNA measured by hybridization methods in the vaccine product was below the requirement (10 ng/dose) specified by

Table 3 Immunogenicity and protective activity of ACAM1000 and Dryvax® in mice and monkeys.

Study	Species (strain)	Vaccine (8 $\log_{10}$ pfu/mL)	No. animals	Immune response				Challenge	Survival
				Cutaneous pock *N* (%)	Neutralizing antibody *N* (%)	Neutralizing antibody titer[a]	IFN-γ secreting cells × 10^{-61}		
1	Mouse (Balb/c)	ACAM1000	10	—[b]	10 (100%)	53	—	Cowpox 10 LD_{50} IN[c]	10/10 (100%)
		Dryvax®	9	—	5 (56%)	14	—		7/7 (100%)
		Sham	5	—	0 (0%)	<10	—		0/5 (0%)
2	Mouse (Balb/c)	ACAM1000	5	—	—	—	—	Vaccinia WR 100 LD_{50} IN[d]	5/5 (100%)
		Dryvax®	5	—	—	—	—		5/5 (100%)
		Sham	5	—	—	—	—		0/5 (0%)
3	Mouse (Balb/c)	ACAM1000	5	—	5 (100%)	92	1450		
		Dryvax®	5	—	3 (60%)	20	1052		
		Sham	5	—	0 (0%)	<20	0		
4	Mouse (Balb/c)	ACAM1000	4	—	—	—	—	Ectromelia 4.3 $\log_{10}$ pfu aerosol	4/4 (100%)
		Dryvax®	4	—	—	—	—		4/4 (100%)
		Sham	4	—	—	—	—		0/4 (0%)
5	Monkey (rhesus)	ACAM1000	6	6 (100%)	5 (83%)	18	—		
		Dryvax®	6	6 (100%)	4 (67%)	25	—		

[a] Geometric mean.
[b] — = not tested.
[c] 5 weeks after vaccination.
[d] 3 weeks after vaccination.

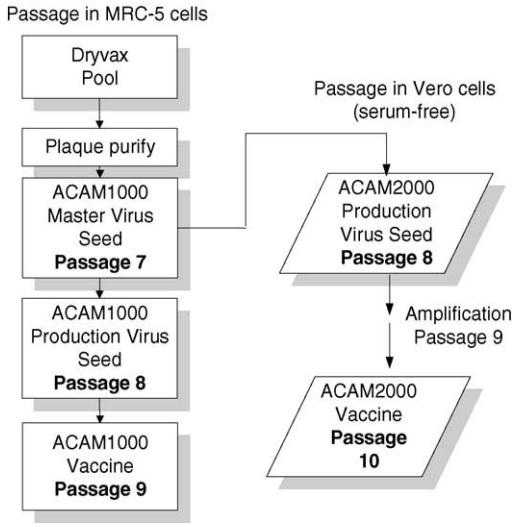

Figure 2 Passage history of ACAM1000 and ACAM2000.

WHO. Size analysis by Southern blot showed that the DNA had been digested into small fragments.

Molecular characterization

Since DNA viruses have very low mutation rates during passage, it was anticipated that the clonal ACAM1000 and ACAM2000 vaccines would be genetically and phenotypically conserved. To confirm this, we fully sequenced the genomes of the ACAM1000 master virus seed (P7, MRC-5 cells) and a representative lot of ACAM2000 vaccine (P10, 3 passages in Vero cells beyond the ACAM1000 master virus seed) (Figure 1). The sequences of ACAM1000 (determined at St. Louis University) and ACAM2000 (determined at CDC) were found to be identical (personal communication, J. Esposito et al., CDC, Dec 30, 2003).

Biological comparison of ACAM1000 and ACAM2000

The neurovirulence profiles of ACAM2000 (P10) and ACAM1000 (P9) vaccines were compared in a lethal dose assay to Dryvax® and the virulent Clone 3 virus recovered from Dryvax®. Groups of eleven 3–4 day-old outbred ICR mice were inoculated with graded doses (0.3 to 3.0 $\log_{10}$ pfu) by the IC route. The median lethal dose (LD_{50}) and 90% lethal dose (LD_{90}) were higher for mice receiving ACAM2000 and ACAM1000 compared to Dryvax® and Clone 3 viruses (Table 5). Survival analysis showed that ACAM1000 and ACAM2000 did not differ from one another, but had significantly longer survival than Dryvax® ($p < 0.05$, Kaplan Meier survival distribution, log rank test) (Figure 1B).

ACAM2000 (P10), ACAM1000 (P9), and Dryvax® were each tested in 5 rabbits for cutaneous virulence following percutaneous inoculation of each virus from 8 to 5 $\log_{10}$ PFU/mL delivered by bifurcated needle, as described above (**Preclinical safety in animal models**). Central lesions and erythema produced by ACAM1000 and ACAM2000 did not differ, but both tended to be slightly less than those evoked by Dryvax® (Figure 3).

Balb/c mice were immunized by scarification at the base of the tail with 6 or 8 $\log_{10}$ PFU/mL of ACAM2000 (P10), ACAM1000 (P9), or Dryvax® and neutralizing antibody and T cell responses determined 3 weeks later. Mean neutralizing antibody responses following vaccination with ACAM1000 and ACAM2000 were higher than following Dryvax® at both the high and low doses, but did not differ significantly ($p < .05$, Wilcoxon rank sum test) (Figure 4A). IFN-γ secreting cells stimulated with vaccinia WR-infected P815 cells were abundant in all treatment groups (Figure 4B). The specificity of the T cell response was also examined using $CD8^+$ splenic lymphocytes from mice immunized with 8 $\log_{10}$ PFU/mL and depleted of B cells, $CD4^+$ cells, macrophages, and NK cells using monoclonal antibody-conjugated magnetic beads and magnetic cell sorting. Strong CD8+ responses were seen in all three treatment groups but not in unimmunized controls, and there were no statistical differences in responses across the vaccine treatment groups (data not shown).

To compare the protective efficacy of immunization with ACAM2000, ACAM1000, and Dryvax®, groups of 5 young adult BALB/c mice were immunized with graded doses (4 to 7 $\log_{10}$ PFU/mL) of the different viruses and then challenged by the IN route 3 weeks later with 100 LD_{50} of vaccinia WR virus. Survival and body weight were recorded daily for 14 days after challenge. Protective efficacy of the 3 viruses tested was similar. The survival times were not statistically different between treatment groups ($p < 0.05$, Kaplan–Meier survival distribution, log rank test). Doses required for protection of 50% of mice from death were also similar for ACAM1000 (5.4 $\log_{10}$ PFU/mL), ACAM2000 (5.6 $\log_{10}$ PFU/mL) and Dryvax® (5.2 $\log_{10}$ PFU/mL).

Clinical trials

Clinical development was initiated with a pilot lot of ACAM1000. The first trial was a randomized, double-blind study in healthy adults aged 18–29 years, and has been reported by Weltzin et al.[10] Briefly, all 30 subjects (100%, 95% CI 88, 100) inoculated with

Table 4 Tests performed to control ACAM2000 for adventitious agents (blank spaces in the table indicate that the test was not performed).

Description of Test	Method	Master Virus Seed (ACAM 1000/2000) P7	Production Virus Seed (ACAM2000) P8	Vaccine lot (ACAM2000) P10
Sterility	Culture for bacteria and fungi	X	X	X
Mycobacteria	42-day guinea pig test	X		
Mycoplasma	Cultivation Method	X	X	X
	Polymerase chain reaction (PCR), agar cultivatable and non-cultivatable	X	X	X
Adventitious Virus (general)	*In vitro* virus assay (Vero, MRC-5, and either HeLa and RK cells or A9 cells)	X	X	X
Adventitious Virus (general)	*In vivo* virus assay (suckling and adult mice, guinea pigs, embryonated eggs)	X	X	X
Adventitious virus (animal)	Bovine viral diarrhea (PCR)	X	X	X[a]
	Bovine viral diarrhea (*in vitro* infectivity)		X	
	Bovine parainfluenza type 3 (PCR)	X	X	X[a]
	Bovine respiratory syncytial virus (PCR)	X	X	X[a]
	Bovine adenovirus (PCR)	X	X	X[a]
	Bovine parvovirus (PCR)	X	X	X[a]
	Porcine parvovirus (PCR)	X		X[a]
	Bovine herpes virus-I (bovine rhinotracheitis) and bovine herpes-IV (PCR)		X	X[a]
	Bovine reovirus (PCR)		X	X[a]
	Rabies virus (PCR)		X	X[a]
	Bluetongue virus (PCR)		X	X[a]
	Bovine polyoma virus (PCR)	X	X	X[a]
Adventitious Viruses (human)	HIV 1 and 2 (PCR)	X	X	X[a]
	Hepatitis B (PCR)	X	X	X[a]
	Cytomegalovirus (PCR)	X	X	X[a]
	Epstein Barr Virus (PCR)	X	X	X[a]
	Human herpesvirus type 6 (PCR)	X	X	X[a]
	Human herpesvirus type 7 (PCR)	X	X	X[a]
	Human herpesvirus type 8 (PCR)		X	X[a]
	Hepatitis C (PCR)	X	X	X[a]
	HTLV I and II (PCR)	X	X	X[a]
	Parvovirus B19 (PCR)	X	X	X[a]
	Reovirus (PCR)		X	X[a]
	JC/BK virus (PCR)		X	X[a]
	SV40 virus (PCR)		X	X[a]
	Coronavirus (PCR)		X	X[a]

Table 4 (*Continued*)

Description of Test	Method	Master Virus Seed (ACAM 1000/2000) P7	Production Virus Seed (ACAM2000) P8	Vaccine lot (ACAM2000) P10
	Human Papilloma Virus (PCR)		X	X[a]
	Hepatitis A virus (PCR)		X	X[a]
	Enterovirus (PCR)		X	X[a]
	Influenza C (PCR)		X	X[a]
	Human parainfluenza types 1, 2, 3 (PCR)		X	X[a]
	Human respiratory syncytial virus Types A and B (PCR)		X	X[a]
	Retrovirus (f-PERT)	X	X	

[a] Performed on three successive lots to demonstrate consistent freedom from adventitious agents.

ACAM1000 and 29 of 30 subjects (97%, 95% CI 83, 100) inoculated with Dryvax® developed a major cutaneous reaction 7 days after vaccination (Table 6). The same proportions of subjects in each treatment group developed neutralizing antibodies ($p = 1.000$, Fisher's exact test). The geometric mean neutralizing antibody titers in the ACAM1000 and Dryvax® groups of 142 and 248, respectively, were not statistically different ($p = 0.181$, ANOVA). T cell responses were measured by cytotoxic T cell assay, IFN-γ ELISPOT, and lymphoproliferation.[10] T cell responses were seen to both vaccines in the vast majority of subjects (Table 6). In the lymphoproliferation assay, ACAM1000 responses were significantly higher than in the Dryvax® group. The adverse event profile of ACAM1000 and Dryvax® were also similar (Table 7).

These clinical results suggested that ACAM1000 was a suitable candidate smallpox vaccine. However, the Phase 1 study had been conducted within 6 months of the events of September 11, and there was grave concern about bioterrorism. The question arose as to how much clinical data should be available with the new vaccine to allow its wider use in an emergency. It was determined in meetings with US Government officials, that clinical data from a minimum of 100 subjects should be available. Therefore, to bring the total number to 100 subjects, a second study was performed. This second Phase 1, open-label study was designed to evaluate the safety, tolerability, and immunogenicity of ACAM1000 smallpox vaccine in 70 adults aged 18 to 29 years, inclusive, who were naïve to smallpox vaccine, and enrolled in the study at a single study center in the United States. Eligible subjects and their household contacts had no contraindications to smallpox vaccination (atopic dermatitis, other active skin diseases, immune deficiency, pregnancy). Safety was assessed by documentation of adverse events, physical examination findings, lymph node assessments, measurements of vital signs, and clinical laboratory tests, including hematology, clinical chemistry, and urinalysis. Eligible subjects who provided informed consent received a single vaccination with ACAM1000. Subjects returned to the clinic on Days 3, 7, 10, 15, and 30 for evaluations and also kept a diary of adverse events and took daily oral temperatures. Fifty-one percent of subjects were male. The majority (87%) were Caucasian. All 70 subjects (100%) experienced

Table 5 Median Lethal Dose (LD_{50}) of ACAM1000, ACAM2000, Dryvax®, and Clone 3 in Mice.

Virus	LD_{50}[a]	LD_{90}[b]
ACAM2000	1.6	2.9
ACAM1000	2.6	>3.3
Dryvax®	0.8	2.2
Clone 3	<0.3	1.1

[a] 50% IC lethal dose (log_{10}) per 0.02 mL inoculum.
[b] 90% IC lethal dose (log_{10}) per 0.02 mL inoculum.

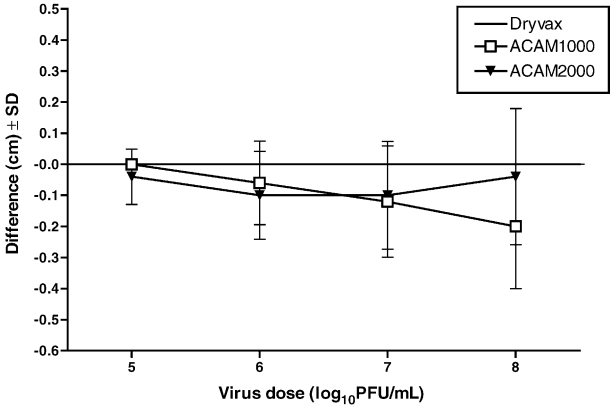

Figure 3 The central lesion resulting from graded doses of ACAM1000 or ACAM2000 was compared to the lesion caused by the equivalent dose of Dryvax® in the same rabbit, and the difference in lesion diameter was determined.

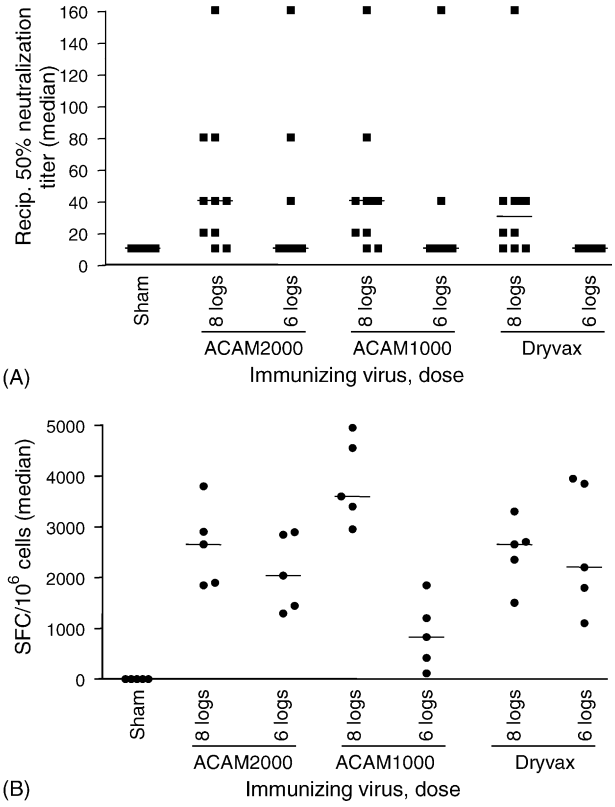

Figure 4 (A) Neutralizing antibody responses following immunization of mice with ACAM2000, ACAM1000, or Dryvax®. (B) T Cell responses following immunization of mice with ACAM2000, ACAM1000, or Dryvax®. Secretion of interferon-γ by splenic lymphocytes stimulated by vaccinia virus *in vitro*.

a successful vaccination (major cutaneous reaction) by Day 7–10 after vaccination. Seroconversion (≥fourfold increase in neutralizing antibody titer by Day 30) was demonstrated in 66 (94%) of the subjects (Table 6). The geometric mean neutralizing antibody titer on Day 30 was 154; the titer varied considerably, ranging from 20 to 20,480. The evolution of lesions and size of the pock lesions were similar to those observed in the first trial, with a papule or vesicle appearing on Day 3, progressing to a pustular lesion by Day 7 and scabbing over in the majority of cases by Day 15. There were no serious adverse events. All 70 subjects (100%) experienced at least one treatment-emergent, expected adverse event during the study (Table 7). Overall, the most commonly reported adverse events were related to the inoculation site. The adverse events were generally mild and did not interfere with the subjects' daily activities.

Clinical development of ACAM2000 commenced with a Phase 1 open-label trial in 100 healthy adults aged 18–29 years old without prior smallpox vaccination. Exclusion and inclusion criteria were the same as for the ACAM1000 study described above. The primary endpoint was the proportion of subjects with a major cutaneous reaction assessed at any time-point from Day 7 (±2) through Day 15 (±2). Fifty-six percent of subjects were male. The majority (89%) were Caucasian; the remaining subjects were African-American (7%), Asian (3%), or Hispanic (1%). The mean age was 23 years, with a range of 18 to 29 years.

Table 6 Immunogenicity, ACAM1000 and ACAM2000, Phase 1 clinical trials.

Statistic	Study number/Study vaccine			
	H-300-001		H-300-003	H-400-008
	ACAM1000	Dryvax®	ACAM1000	ACAM2000
Number of subjects	30	30	70	100
Major cutaneous reaction				
No. (%)	30 (100)	29 (97)	70 (100)	99 (99)
95% Confidence interval	88, 100	83, 100	95, 100	95, 100
Maximum lesion size (mm)				
Central lesion, mean (±SD)	13.4 (1.87)	12.6 (2.37)	10.9 (1.76)	10.3 (1.83)
Erythema, mean (±SD)	25.8 (8.52)	30.7 (16.09)	22.8 (15.79)	35.7 (17.32)
Seroconversion, neutralizing antibodies				
No. (%)	30 (100)	29 (97)	66 (94)	96 (96)
Neutralizing antibodies, Geometric Mean Titer	142	248	154	225
T cell response No. pos/tested (%)				
Cytotoxic T lymphocyte	24/29 (80)	26/30 (90)	Not tested	Not tested
γ-Interferon ELISPOT	29/29 (100)	29/30 (97)		
Lymphoproliferation	28/29 (97)	21/30 (70)		

Table 7 Treatment-emergent adverse events (AE) reported by ≥5% of subjects.

	Study No. and treatment			
	H-300-001		H-300-003	H-400-008
	ACAM1000 (n = 30) n (%)	Dryvax® (n = 30) n (%)	ACAM1000 (n = 70) n (%)	ACAM2000 (n = 100) n (%)
Subjects with at least 1 AE	30 (100)	30 (100)	70 (100)	100 (100)
Injection site erythema	29 (97)	28 (93)	70 (100)	100 (100)
Injection site pruritus	19 (63)	15 (50)	62 (89)	97 (97)
Injection site pain	24 (80)	27 (90)	50 (71)	67 (67)
Lymph node or axillary pain	22 (73)	19 (63)	50 (71)	67 (67)
Headache NOS	18 (60)	18 (60)	36 (51)	46 (46)
Fatigue	6 (20)	9 (30)	28 (40)	45 (45)
Lymphadenopathy	5 (17)	7 (23)	16 (23)	40 (40)
Injection site inflammation	24 (80)	25 (83)	58 (83)	37 (37)
Myalgia	8 (27)	9 (30)	22 (31)	38 (38)
Malaise	5 (17)	4 (13)	17 (24)	38 (38)
Feeling hot	11 (37)	13 (43)	11 (16)	31 (31)
Rigors	5 (17)	6 (20)	12 (17)	18 (18)
Nausea	1 (3)	2 (7)	11 (16)	13 (13)
Diarrhea NOS	2 (7)	1 (3)	7 (10)	11 (11)
Body temperature increased	1 (3)	5 (17)	4 (6)	9 (9)
Cough	1 (3)	2 (7)	0	5 (5)
Upper respiratory tract infection	5 (17)	3 (10)	3 (4)	4 (4)
White blood cells urine positive	3 (10)	5 (17)	5 (7)	4 (4)
Pharyngitis	3 (10)	3 (10)	4 (6)	4 (4)
Nasal congestion	0	4 (13)	1 (1)	3 (3)
Rash NOS	3 (10)	4 (13)	6 (9)	2 (2)
Injection site burning	3 (10)	1 (3)	1 (1)	1 (1)
Abdominal pain upper	1 (3)	2 (7)	0	1 (1)
Dizziness	1 (3)	2 (7)	2 (3)	0
Application site irritation	1 (3)	2 (7)	0	0
Neck stiffness	2 (7)	0	0	0

NOS = Not otherwise specified.

Ninety-nine percent of the subjects experienced a successful vaccination in this study (Table 6). Of the 99 subjects who experienced a major cutaneous reaction, 9% had a major cutaneous reaction by Day 3, and the rest experienced a major cutaneous reaction by Day 7. The progression of the cutaneous reaction (Figure 5) and its size and appearance were similar to those observed in the trials of ACAM1000 (data not shown). The great majority (96%) developed ≥fourfold increases in neutralizing antibodies. The geometric mean neutralizing antibody titer on Day 30 was 225. Four (4%) of 100 subjects did not have a fourfold increase in neutralizing antibody titer on Day 30. However, these 4 subjects all had a major cutaneous reaction by Day 7. This illustrated that local replication in the skin and development of a pock is not invariably accompanied by a systemic humoral immune response detectable by a 50% plaque-reduction neutralization test.

One subject experienced a serious adverse event, a single new onset seizure on Day 8; this event was considered by the Investigator to be remotely related to the study vaccine. All 100 (100%) subjects experienced at least 1 treatment-emergent adverse event during the study. The most commonly reported treatment-emergent adverse events were related to the vaccination site and associated lymphadenitis (Table 7), and the majority of adverse events reported were assessed as mild or moderate in intensity. No notable changes from Screening to

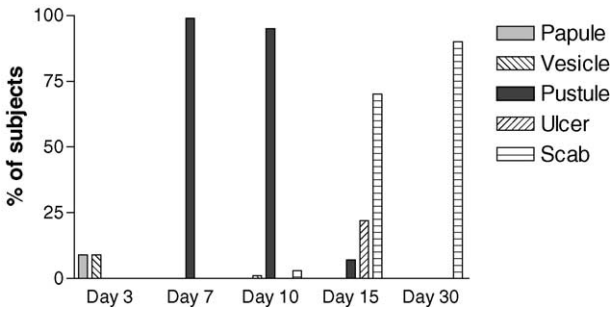

Figure 5 Progression of cutaneous lesion in healthy adults without previous smallpox vaccination who were inoculated with ACAM2000.

Day 15 were seen in any hematologic or clinical chemistry parameter. Minimal changes in mean oral temperature were observed. Elevated temperature was reported as an adverse event for 9 (9%) subjects. Of these 9 subjects, 1 subject with concurrent streptococcal pharyngitis had a body temperature $>38.9\ ^{\circ}C$ during the study. For the remaining subjects, body temperature was $<38.9\ ^{\circ}C$ at all time-points assessed. Lymph node enlargement and/or tenderness was present on at least 1 post-vaccination time-point for 61 (61%) of 100 subjects.

Discussion

It has long been recognized that vaccinia virus represents a heterogeneous swarm of virus subpopulations that differ biologically and molecularly.[11] This variation became especially important as vaccinia was developed as a live vector for foreign genes. Plaque-purified vaccinia virus lines were shown to differ significantly in neurovirulence for mice, in their ability to evoke immune responses against the inserted gene product, and in their Hind*III* restriction maps.[12,13] From virus stocks that underwent serial passages, variants could be recovered that contained deletions within or extending beyond the inverted terminal repeat regions,[14–17] some of which were as large as 9 MDa (~5% of the genome). The variant viruses often exhibit reduced infectivity and reduced virulence for mice.[18] Similar deletion variants have been reported for other orthopoxviruses, including cowpox and monkeypox. It is not surprising, therefore, that we found biological and molecular heterogeneity among 6 clones derived from Dryvax®, with some clonal subpopulations (e.g. Clone 3) having dramatically higher virulence[10] and changes at the genomic level (Esposito J pers. comm.). Clone 3 derived from Dryvax® was shown to have an altered Hind*III* restriction map[10] and to contain a deletion at the right terminus and hundreds of mutations compared to the attenuated ACAM2000 (Clone 2). These observations were important when considering development of the second generation cell culture vaccine. The production of vaccine at a large scale in cell culture would put selective pressure on the virus, resulting in a different ratio of virion subpopulations in the end product compared to that in the original calf-skin vaccine. Indeed, we found that passage of uncloned Dryvax® in MRC-5 cells yielded a vaccine candidate that was unacceptably virulent. Such selective pressures could be inconsistent across vaccine lots made at large scale, with resulting variability in biological phenotype, and lot-to-lot differences in tests used to release the product.

The clinical relevance of virus heterogeneity in first generation smallpox vaccines is uncertain. However, there is reason to believe that a well-defined clonal product has advantages. Ehrengut et al.[19,20] recovered vaccinia virus from patients who had dermatologic (vaccinia ulcer) or neurological complications. The viruses isolated from the affected patients differed from the parental vaccine in being resistant to high temperature, growing to higher titer in cell culture, producing larger plaque sizes, and causing marked necrosis in rabbit skin after intradermal inoculation. Similarly, Vilesova et al.[21] studied 6 virus isolates from cerebrospinal fluid and brain tissue of patients with post-vaccinal encephalitis. These strains were also thermostable, exhibited larger plaque size in cell culture, and were more pathogenic for chick embryos and more dermovirulent in rabbits than the parental vaccine. These data suggest that selection of a virulent virus subpopulation can occasionally occur during replication in the vaccinated host, leading to neurological or dermal complications.

The degree of neurovirulence for suckling mice was used by Marrenikova[21,22] to distinguish vaccine strains with low, moderate, or high pathogenicity. This classification by toxicity for mice correlated with the incidence of post-vaccinal encephalitis in human populations exposed to the corresponding vaccine strains[22–24]. For example, vaccines with high mouse pathogenicity, such as the Temple of Heaven and Tashkent strains, were associated with high incidence rates of post-vaccinial encephalitis, whereas the New York City Board of Health (NYCBH) strain (e.g. Dryvax®) was associated with the lowest rates of this complication. The Lister strain, widely used in Europe and elsewhere, is of moderate mouse pathogenicity, and may have caused a higher rate of neurological complications than NYCBH in humans. We showed that ACAM1000 and ACAM2000 were significantly less neurovirulent for mice and monkeys than the parental Dryvax® virus, presumably because it contains neurovirulent virus subpopulations exemplified by Clones 1, 3 and 5. It is logical to assume, based on the discussion above, that ACAM2000 will be less likely to cause post-vaccinal encephalitis in humans. However, since the incidence of this complication following Dryvax® vaccination is so low (in the range of 1–5 per million[1,2]), it would not be possible to prove this contention in clinical trials. Moreover, the pathogenesis of post-vaccinal encephalitis is still uncertain, with controversy regarding the role of neuroinvasion and direct viral injury, particularly in cases of post-vaccinial encephalitis in adults. Vaccinia virus has been isolated from CSF and brain,[25,26] suggesting that the virus invades the central nervous system in humans.

The events that follow could have an immunopathological component, leading to destruction of myelin. Animal models (mice, monkeys) mimic the early-onset edematous form of meningoencephalitis observed more frequently in infants,[27,28] but do not show perivenous demyelination typical of the adult form of the disease, which clinically resembles acute disseminated encephalomyelitis (ADEM) and suggests a parainfectious (immunopathological) disease process.

Phase 1 clinical trials of ACAM1000 and 2000 indicate that the original goal of producing a second generation vaccine that closely matched the safety and immunogenicity of calf-skin vaccine (Dryvax®) was met. The cutaneous, antibody, and T cell responses in primary vaccinees were similar to those elicited by Dryvax®. The appearance (Figure 5) and size of the cutaneous lesion and pattern of virus shedding from the vaccination site were also similar. Phase 2 trials in naïve and previously vaccinated subjects have been completed to define the dose response, and to extend safety and immunogenicity data.[29] Phase 3 clinical trials are in progress. The principal finding of concern in Phase 2 and 3 trials involving larger numbers of subjects has been the discovery that smallpox vaccines are associated with inflammation of the myocardium and pericardium (myopericarditis).[30,31] Although myopericarditis had been reported previously, it had not been recognized as a frequent complication of vaccination. In clinical trials of ACAM2000 and Dryvax®, myopericarditis has been associated with both vaccines, and appears to be an immunopathological response to vaccinia infection. The military experience with Dryvax® suggests that the incidence of myopericarditis detected by passive surveillance approximates 1:8000.[30] A significantly higher incidence is being detected in our Phase 3 clinical trials, since subjects are evaluated prospectively with provoked questions for cardiac symptoms and with serial electrocardiograms and serum enzyme tests. Fortunately, the cardiac adverse events appear to be self-limited. These data will be reported in future publications.

In summary, ACAM2000 appears to be a suitable vaccine for prevention of smallpox. The similar immune responses in humans and protective activity in animals to the currently licensed Dryvax® vaccine (which was used in the global program to eradicate smallpox) provide confidence that the vaccine will protect against variola. The new vaccine has advantages over first generation vaccines, since it has been produced to modern manufacturing and control standards, is free from adventitious agents (Table 5), and does not contain subpopulations of virus with undesirable virulence properties. It is also a 'very low pathogenicity' strain, being less neurovirulent for animals than Dryvax®. The latter property may be associated with a lower propensity to cause post-vaccinial encephalitis, but this proposition cannot be proven without very wide-scale use of the vaccine to evaluate incidence rates.

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