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STUDIES ON THE STABILITY, PRODUCTION AND MICROENCAPSULATION OF A RECOMBINANT HUMAN ADENOVIRUS-RABIES VACCINE

A Thesis submitted to the
School of Graduate Studies
University of Ottawa

In Partial Fulfillment of the Requirement for the Degree of
Master of Science
Department of Microbiology and Immunology
Faculty of Medicine

by

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UNIVERSITY OF OTTAWA

Dedicated to the memory
of my brother Kamal

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ABSTRACT

The recombinant human adenovirus type 5 (rHAd5-RG1) tested in this study contains the rabies virus glycoprotein gene. This virus is being considered as a possible substitute for the attenuated rabies virus in the oral immunization of wildlife in Ontario. This study examined the stability of the virus indoors and outdoors, and tested technically simple ways of concentrating the virus and its microencapsulation. The findings were as follows:

1) The virus survived well in both Earle's balanced salt solution (EBSS) and a commercial egg yolk-containing stabilizer when stored for 16 days either at room temperature (23±2°C) or at 4°C. Even though virus survival was somewhat better in the stabilizer, the use of such material in the vaccine would add to the cost and quality control. In view of this, the virus could be stored without the stabilizer, preferably under refrigeration.

2) In comparative tests on virus suspensions stored at 37°C, the recombinant showed the same thermostability as its parent (E3 deletion) and a wild-type human adenovirus type 5. This indicates that the genetic manipulation of the recombinant has not altered its thermostability.

3) The recombinant survived lyophilization and was able to retain its infectivity well in the lyophilized state when stored at either room temperature or at 4°C. The use of a lyophilized vaccine may eliminate the need for refrigeration during its storage and shipment.

4) The virus was suspended in fox or skunk feces and placed on metal

disks to determine the influence of relative humidity (RH) on its survival at room temperature. The virus remained viable for at least 3 days. This points to the care that should be exercised in handling virus contaminated surfaces and objects.

5) The virus was suspended in 5% fetal calf serum and dried on metal disks in order to test the efficacy of commonly used hard-surface chemical disinfectants against it. The products evaluated were 70% ethanol, 2% glutaraldehyde, 1% domestic bleach (sodium hypochlorite) and Lysol Disinfectant Spray (80% ethanol) with a contact time of 10 min at room temperature. All of them were able to inactivate the virus suggesting that any one of them can be used for the decontamination of surfaces and objects where the virus is being handled.

6) To determine the stability of the virus under outdoor conditions, it was placed in blister packs, encased in the bait and kept either in the shade or in the sun. These field exposures were carried out in the falls of 1990 and 1991. There was a half-$\log_{10}$ drop in virus infectivity over 32 days in both the trials, indicating the relative stability of the virus outdoors. Incorporation of proteinaceous substances in the virus suspension during outdoor exposure did not increase virus stability.

7) Freon and a number of mild detergents, alone or in combination, were tested to dissociate cell-associated virus. Sodium deoxycholate alone and a mixture of NP-40 and Triton X-100 increased infectious virus yield 5.5- and 5.9-fold, respectively, when compared to untreated virus infected cells.

8) Polyethylene glycol 8000 (PEG) and polyacrylamide (Aquacide IV) were tested for virus concentration. PEG proved to be more efficient with a virus recovery of up to 66% and a ten-fold reduction in volume. This concentration procedure is relatively simple and inexpensive and could be scaled up for commercial production of the vaccine.

9) Cellulose acetate phthalate (CAP) and sodium alginate were tested for the microencapsulation of the virus. Although both the coatings appeared to release the encapsulated bovine albumin or ^{35}S-methionine labelled virus in a pH dependent manner, the sodium alginate procedure was found to be simpler and gentler for virus infectivity.

The findings of this study show that the recombinant virus has the potential for release into the environment and there is no evidence that the virus will rapidly decay under the outdoor conditions experienced during the fall season. The virus can probably be microencapsulated and packaged for oral delivery. However, more studies are needed to assess the actual immunizing potential of these microencapsules. The use of detergents for increasing the virus yield has potential.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
°C	degree Celsius
Ca^{++}	calcium ion
CAP	cellulose acetate phthalate
CHAPS	3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate
cm	centimeter
cm^2	centimeter squared
CPE	cytopathic effect
CO_2	carbon dioxide
DP	diethyl phthalate
EDTA	ethylenediaminetetraacetic acid
EBSS	Earle's balanced salt solution
EMEM	Eagle minimal essential medium
FCS	fetal calf serum
hr	hour
HDCV	human diploid cell vaccine
K_i	inactivation rate
L	liter
Mg^{++}	magnesium ion
$MgCl_2$	magnesium chloride
mg	milligram
min	minute
mL	milliliter
MOI	multiplicity of infection
NaOH	sodium hydroxide
nm	nanometer
PEG	polyethylene glycol
PBS	phosphate buffered saline
PFU	plaque forming units
ppm	parts per million

rpm	revolutions per minute
RH	relative humidity
rHAd5-RG1	recombinant human adenovirus type 5 with the ERA rabies glycoprotein gene
s	second
SAD	Street Alabama Dufferin
$TCID_{50}$	tissue culture infectivity dose-50%
µg	microgram
µL	microliter
USP	United States Pharmacopia
v/v	volume per volume
w/v	weight per volume

INTRODUCTION

REVIEW OF THE LITERATURE

1. RABIES

1.1 Introduction

Rabies is one of the oldest known and most feared of the human diseases. It is prevalent in many regions of the world and is caused by the rabies virus which infects humans and other warm-blooded animals producing an acute encephalitis that is almost always fatal.

The history of rabies has been documented by a long series of observations relating to prevention, treatment and laboratory diagnosis of the disease. In 1804, Zinke first transmitted rabies to a healthy dog by inoculating it with saliva from an infected animal. This observation subsequently led to the institution of dog control and muzzle laws that resulted in the elimination of rabies from Denmark, Norway and Sweden by 1826. In 1879, Galtier was the first to use rabbits as laboratory hosts for the virus. This allowed Pasteur to develop fixed rabies viruses which he grew in rabbit spinal cord. The dried spinal cords containing the virus were first used in 1885 to immunize a boy against rabies. This then paved the way for further development of anti-rabies vaccines (Shope, 1989).

1.2 Clinical features of rabies

Human rabies cases occur mostly by animal bites and rarely by nonbite exposures. However, nonbite exposures such as licks and scratches may result in the contamination of an open wound or mucous membrane. Studies have shown that inhalation of virus-laden aerosols can lead to rabies as documented in

individuals visiting bat infested caves (Constantine, 1962) and laboratory workers inhaling aerosols from virus-contaminated tissues (Winkler *et al.*, 1973; Conomy *et al.*, 1977). The only documented mode of person-to-person transmission of the rabies virus has been through corneal transplants in which the recipients developed acute and fatal encephalitis that was later determined to have been caused by the rabies virus (Honff *et al.*, 1979; Gode and Bhide, 1988). Studies in animals indicate that the virus can be transmitted by the oral and nasal routes as well (Fischman and Ward III, 1968; Hronovosky and Benda, 1969; Charlton and Casey, 1977).

In humans, the incubation period of the disease varies with 99% of the cases occurring within one year after exposure to the virus. The average length is between 20 and 90 days (Baer *et al.*, 1990; Bernard and Hattwick, 1985). Several factors are thought to contribute to the variation in the incubation period. These include:

(a) *the site of the bite*: the incubation period after a bite on the head is shorter than one on the extremities. This can be attributed to the proximity to the central nervous system (Nikolitsch, 1958).

(b) *the severity of the bite and the quantity of virus inoculated* (Sikes, 1962; Dupont and Earle, 1966).

(c) *the age of the individual*: the incubation period in children is shorter than that in adults (Dupont and Earle, 1966).

(d) *the immunological and nutritional status of the infected individual*: the use

of immunosuppressive agents (corticosteroids) and malnutrition can decrease the incubation period (Enright, 1974; Dupont and Earle, 1966).

After the incubation period in humans, a period of nonspecific symptoms (prodromal phase) that include chills, fever, malaise and cough begins and continues for 2 to 10 days. It is during this phase that the virus is invading the central nervous system. Symptoms often noted during this phase are pain and other paraesthesia such as burning, numbness, tingling or itching at the bite site. These are the result of viral replication in the spinal cord (Baer *et al.*, 1990).

Following the prodromal phase is the acute neurologic phase which is characterized by nervous system dysfunctions. In about 80% of patients, this manifests itself as furious rabies during which the patient exhibits hallucinations, hydrophobia, aerophobia, increased anxiety and agitation. The other 20% get paralytic (dumb) rabies characterized by a symmetrically ascending paralysis that progresses to complete paralysis. The acute neurologic period ends after 2 to 7 days, with coma or death from respiratory arrest (Bernard and Hattwick, 1985).

In animals, a similar clinical manifestation and variable incubation period have been noted. In dogs, the incubation period varies from 9 to 90 days. The prodromal phase is characterized by fever, irritability, and restlessness. At this stage the animal has an increased tendency to bite. After 5 to 10 days, the animal enters a paralytic stage with paralysis of the whole body, coma and finally death (Jawetz *et al.*, 1989).

1.3 Rabies virus

Rabies virus is the prototype of a small group of serologically related viruses in the genus Lyssavirus, within the family Rhabdoviridae. Other members of this group are the Lagos bat, Mokola, Kotonkan, Duvenhage and Obodhiang viruses (Shope, 1989; Baer *et al.*, 1990).

The hallmark of rhabdoviruses is their bullet-shaped morphology. Rabies virus particles have an average length of 180 nm (range 130-300 nm) and an average diameter of 75 nm (Hummeler *et al.*, 1967; Vernon *et al.*, 1972; Wunner, 1985).

The viral genome consists of a single-stranded, non-segmented RNA with a molecular weight estimated at 4.6×10^6 daltons. The isolated RNA is not infectious indicating that it has a negative-sense polarity and therefore transcription of the virion RNA into complementary mRNA is required before viral replication can occur. Various techniques, including transcription mapping, have shown that the genome encodes five structural proteins. Using a system that couples transcription and translation, the genes were identified in the 3′ to 5′ order as follows: N (nucleoprotein), P (phosphoprotein), M (matrix), G (glycoprotein) and L (polymerase) (Flamand and Delagneau, 1978; Tordo and Poch, 1988; Wunner, 1985).

1.4 Rabies virus pathogenesis

The pathogenic mechanism used by the virus is still poorly understood. Data suggest that the virus enters striated muscle cells, as might occur during an

animal bite. The virus then undergoes an initial period of replication which is believed to be the amplification step (Baer *et al.*, 1965; Baer and Cleary, 1972; Murphy *et al.*, 1973; Rupprecht, 1987). From here the virus enters the peripheral nervous system through the motor end plate (Dean *et al.*, 1963), a process thought to be mediated by the acetylcholine receptor (Baer *et al.*, 1968; Murphy *et al.*, 1973; Lentz *et al.*, 1982). This was suggested after studies showed the co-localization of the viral antigens at this receptor (Lentz *et al.*, 1982; Murphy, 1985) and an inhibition of this co-localization by the acetylcholine inhibitors, alpha-bungarotoxin and d-tubocurarine (Rupprecht, 1987). There is evidence however to suggest that the virus can bind to various components of the cell membrane including neuramic acid, carbohydrate moieties and phospholipids (phosphatidyl serine and gangliosides) (Hummeler *et al.*, 1967; Tsiang, 1988; Charlton, 1988). Recently, Shankar *et al.*, (1991) observed that a fixed rabies virus (CVS) is capable of entering the central nervous system without any prior replication in the striated muscle cells.

Once in the peripheral nerve cells, the virus progresses centripetally by means of passive retrograde axoplasmic flow at a rate of 50 to 100 mm/day through the spinal ganglia to the spinal cord and then to the central nervous system. The virus is thought to spread between neurons by budding from post-synaptic membranes of infected cells and the simultaneous uptake by a presynaptic axon terminal (Tsiang *et al.*, 1991). Within the brain, the efficiency of the virus spread has been shown to correlate with neurovirulence, with the street

virus spreading faster than a fixed virus; street rabies virus is essentially a wildtype virus isolated from a rabies case, while the fixed virus is a laboratory passaged virus (Jackson, 1991). Subsequent centrifugal viral spread from the central nervous system to a variety of tissues and organs including the cornea, pancreas and salivary glands has been noted (Schnidler, 1961; Dean *et al.*, 1963; Baer *et al.*, 1990).

1.5 Rabies vaccination

The control of urban rabies in North America is due mainly to the institution of mandatory vaccination of domestic animals with either the modified live vaccine or the inactivated cell culture origin virus vaccine (Bunn, 1988). In humans, only individuals at high risk are immunized. These include laboratory personnel working with the virus, veterinarians and animal handlers. The human diploid cell vaccine (HDCV) currently used in humans has proved to be very effective in inducing neutralizing antibodies to the virus and it lacks the capability of inducing the allergic reactions that were observed in the vaccine of neural tissue origin (Baer *et al.*, 1990; Macfarlan, 1988). The HDCV is derived from the supernatant from MRC-5 human embryo fibroblast cell culture that is infected with the rabies virus. Each dose of the vaccine contains the virus (inactivated by beta-propiolactone) as well as human albumin, phenolsulfophthalein, and neomycin sulfate. The vaccine is used for both pre- and post-exposure prophylaxis (Shope, 1989; Murphy and Chanock, 1990).

1.6 Rabies virus epidemiology

Rabies is essentially a disease of animals with the epidemiology of human rabies closely paralleling the epizootiology of animal rabies. Two cycles of animal rabies exist: urban rabies which is maintained by domestic animals and sylvatic rabies which is maintained by the wildlife. Urban rabies is the most common form of rabies in regions of Africa, Asia and South and Central America. Sylvatic rabies is common in regions of Europe and North America (Fernandes and Arambuto, 1985; Blancou, 1988; Shope, 1989). The major wildlife vector of slyvatic rabies in these regions is the red fox (Blancou, 1988; MacInnes *et al.*, 1988).

In North America several other wildlife species along with foxes are involved in the enzootic. These includes skunks, raccoons and insectivorous bats (Blancou *et al.*, 1991). However, different species predominate in various areas. As such six geographically areas were designated. A large part of central USA and Canada from lower Alberta, Saskatchewan and Manitoba to the Rio Grande in Texas reports most of terrestrial wildlife cases in the striped skunk. The Middle Atlantic and southeastern states cases are associated with raccoons. The red fox is associated with cases in New York, Quebec and Ontario, while the arctic fox is associated with cases in Alaska and the Northwest Territories. Within a given geographic area, it is the major host species that is responsible for maintaining the enzootic. Other species may become infected but they do not contribute to maintaining the disease (Smith, 1989). The reasons for the association of a particular wildlife species with the enzootic in a given geographic area is

unknown. However, the use of monoclonal antibodies have led to the identification of several antigenic variants of the street virus within the various geographic areas. The restriction of an enzootic to a limited area could be related to the genetic selection of new virus strains which can develop after serial passage in a target species. This could in turn contribute to the susceptibility, incubation period, length and type of clinical signs and subsequent viral excretion in the saliva (Ramsden and Johnston, 1975; Webster *et al.*, 1985; Smith *et al.*, 1986; Blancou *et al.*, 1991; Smith, 1989). Studies of the distribution of rabies virus in nature have paved the way for the oral immunization of the wildlife.

1.7 Oral vaccination of wildlife

One of the largest outbreaks of wildlife rabies ever reported was in the late 1940's and early 1950's among arctic foxes in northern Canada. The disease spread southward to affect the red fox in most regions of the country including the prairies and central Canada in the middle and late 1950's. This led to massive poisoning of the foxes with strychnine and increased trapping of wild animals in an attempt to control virus spread. However, this could not halt the spread of the disease (MacInnes, 1988). Other more practical methods were needed to control the spread. In 1971, Baer and colleagues (1971) first reported oral vaccination of foxes with an attenuated rabies virus. This led to the search for the most efficacious vaccine (Table 1). Many candidate rabies viruses were tested and finally an attenuated rabies virus strain SAD (Street Alabama Dufferin) was isolated and found to be more immunogenic and less pathogenic than the other

Table 1. Requirements for a live rabies vaccine for the immunization of wildlife (Wandeler *et al.*, 1988)

- The vaccine should:
- orally immunize the target animals.
- not be pathogenic for the target species, non-target species and humans.
- not be excreted.
- not revert to virulent state easily.
- be free from contaminating organisms.
- be stable under environmental conditions for a short period, but not persist in the environment.
- be inexpensive and easy to produce.
- contain at least one genetic marker.

attenuated strains (Nicholson and Bauer, 1981; Black and Lawson, 1973; Koprowski, 1989). The first field trial using the SAD virus was carried out by Steck and Wandeler in Switzerland in 1978 (Steck *et al.*, 1982; Wandeler *et al.*, 1988). This proved to be very successful and rabies was eliminated from most regions of the country. Similar results were obtained from other west European countries including the Federal Republic of Germany, Italy, Austria and France (Baer, 1988; Wandeler *et al.*, 1988; Schneider *et al.*, 1988).

The success of the attenuated rabies vaccine in European countries prompted the Ontario government to initiate similar field trials. A variant of the SAD strain was constructed: the ERA-SAD strain. This virus had proved to be very effective in laboratory studies conducted in foxes (Lawson *et al.*, 1976; Lawson *et al.*, 1982; Lawson *et al.*, 1989; Tolson, 1987). It was subsequently used for the first field trial in Ontario in 1985 (MacInnes, 1988; Lawson *et al.*, 1988) and was found to be relatively unsuccessful. The ERA strain was ineffective in skunks and raccoons which are major reservoirs of rabies in the province (Rupprecht *et al.*, 1990). Furthermore, there was a reduced potency of the vaccine when placed in the field and it lacked gastric acid stability, thus restricting its ability to induce an intestinal mucosal immune response (Tolson, 1987).

The advent of recombinant DNA technology prompted workers to design vaccines using molecular methods. This led to the subsequent construction of recombinant viral vaccines (Table 2). This approach enables a viral vector to function as a vaccine. This technique was applied to vaccinia virus, since a region of this virus was identified as nonessential for viral replication. Initially, a gene of interest which encodes a surface protein for a pathogen (eg. rabies glycoprotein) is cloned into a plasmid which contains some homologous sequences to the nonessential regions of the virus. The recombinant plasmid and the wild type virus are transfected into mammalian cells in culture. This results in homologous recombination between the wild type virus and the recombinant plasmid, thus allowing for the formation of a recombinant virus, which can be

Table 2. General characteristics of a live (recombinant) vaccine

ADVANTAGES
- Presence of only the necessary epitopes for immunization.
- Induces both cell mediated and humoral immunity.
- Relative lack of virulence.
- Possible multiple insertion of genes, thus potential immunity against multiple pathogens.
DISADVANTAGES
- Possible detrimental cross reaction with other strains through recombination.
- Possible expanded tissue tropism and host range for the vector.
- The presence of existing neutralizing antibodies against the vector may neutralize the virus, thus preventing an immune response against the desired gene.

selected by a genetic marker, usually an antibiotic resistance gene (Murphy and Chanock, 1990).

Blancou and colleagues (1986) reported the construction of a recombinant vaccinia virus containing the rabies virus glycoprotein. Other investigators constructed similar vaccinia vectors with the cloned rabies glycoprotein (Esposito *et al.*, 1988; Rupprecht *et al.*, 1986; Tolson, 1988). The results were initially overwhelming with high levels of expression of the rabies glycoprotein along

with high titers of neutralizing antibodies in laboratory animals and foxes. However, further studies found that the vaccinia vaccine was not effective in skunks and raccoons, and was found to be unstable at the gastric acid pH (Tolson *et al.*, 1988).

Another DNA virus, the adenovirus has been shown to be effective in expressing cloned foreign genes (Prevec *et al.*, 1989; Graham, 1990).

2. HUMAN ADENOVIRUS

2.1 Introduction

Adenoviruses were first isolated from cultured human adenoid tissues in 1953 (Rowe *et al.*, 1953) and later from patients with acute respiratory disease (Hilleman *et al.*, 1954). There are currently 47 serotypes of human adenovirus that have been described producing asymptomatic to mild infections although severe disease has been observed in immunosuppressed patients. The human adenoviruses, in particular types 2, 5, and 12 have been the most thoroughly studied and have provided insights into the biology of DNA replication, transcription, RNA processing and protein synthesis in mammalian cells (Horwitz, 1990).

2.2 Biology of adenoviruses

Adenoviruses are nonenveloped icosahedral viruses 60-80 nm in diameter (Maizel Jr *et al.*, 1968). The capsid consists of 252 capsomeres with 12 penton capsomeres residing in the 12 vertices of the icosahedral capsid and 240 hexons occupying the other positions in the structure. Each of the pentons contains a base

on the surface of the capsid and a fiber projecting from the base which is surrounded by five hexons (Morgan *et al.*, 1969; Winters and Russell, 1971).

The core comprises a 36 kb linear double stranded DNA with an inverted terminal repeat that varies from 100-140 base pairs between serotypes. Located at the 5' end is a covalently linked terminal protein that stabilizes the DNA structure and facilitates viral replication (Horwitz, 1990).

Adenoviruses belong to the family Adenoviridae which contains two genera: Mastadenoviruses which infect mammals and Aviadenoviruses which infect avians (Porterfield, 1989). Human adenoviruses are divided into 7 groups (A-G) based on their physical, chemical and biological properties. Group C contains human adenovirus type 5 which is the virus involved in this study.

Infection of permissive cells by human adenoviruses leads to a productive infection (Chardonnet and Dales, 1970b), while semipermissive or nonpermissive cells may undergo transformation (Lucas and Ginsberg, 1972; Strohl and Schlesinger, 1965a). To initiate an infection, the viral fibers attach to receptors located on the cell plasma membrane (Levine and Ginsberg, 1967; Philipson *et al.*, 1968; Lonberg and Philipson, 1969). This leads to a rapid internalization using receptor mediated endocytosis (Sussenbach, 1962; Philipson *et al.*, 1968). Varga *et al.*, (1991) reported that 50% of the surface-bound viruses are internalized within 6 to 7 minutes. Upon entry into the cell, endosomes containing viruses undergo a decrease of intravesicular pH by a proton pump, provoking the release of altered virions into the cytoplasm (Sussenbach, 1967; Russell *et al.*, 1967, Pereira

et al., 1959). The virions are then transferred to the nucleus by association with microtubules (Dales and Chardonnet, 1973; Varga *et al.*, 1991) where uncoating ensues and transcription commences by a cellular DNA-dependent RNA polymerase II (Chardonnet and Dales, 1970a; Green and Daesch, 1961; Green and Pina, 1963). Transcription occurring before viral DNA synthesis is designated as early and begins by two hours after infection. The early (E) transcripts encode six proteins that are generated by a series of splicing mechanisms which is regulated in a temporal fashion. Transcription of the E1A gene is necessary before the other early genes are transcribed since it acts as a trans-activator. Also E1A has been shown to possess transformation capacity in nonpermissive cells (Houweling *et al.*, 1980; Nevins, 1987).

Viral DNA synthesis is initiated by six to ten hours postinfection and occurs in the nucleus requiring both cellular and viral proteins. The process begins at both ends of the inverted repeats with the viral DNA polymerase binding to the terminal protein and proceeding in the 5′ to 3′ direction until the parental strand is copied (Kelly Jr, 1984).

Transcription of late mRNA is also initiated after the onset of viral DNA synthesis. The late (L) genes code for structural proteins and are controlled by the major late promoter (Ishibashi and Maizel Jr, 1974a; Horwitz, 1990). One of the more important of the structural proteins is the polypeptide IX which controls the thermostability of assembled virions and completeness of the packaging of full-sized DNA into capsids (Ghosh-Choudhury *et al.*, 1987). Upon synthesis of the

late proteins, maturation of virus particles occurs in the nuclei of infected cells. The maturation process has been shown to be inefficient with only 10% of the viral DNA being encapsidated (Green *et al.*, 1967; Hayashi and Russell, 1968; Horwitz *et al.*, 1969; Velicer and Ginsberg 1970; Ishibashi and Maizel Jr, 1974).

2.3 Adenovirus infections in humans

Adenoviruses infect the epithelial cells of the pharynx, conjunctivae, and small intestine and cause pharyngitis, conjunctivitis and diarrhea, respectively. The group C viruses can persist in the adenoids and tonsils and account for 5% of the acute respiratory disease in young children (Van Der Veen, 1963).

2.4 Adenovirus vectors and vaccines

Among the human adenoviruses, the potential use of adenovirus type 5 as a live vector is considered for several reasons: first, its genetic material has been widely studied and characterized; second, they are easy to grow and manipulate and exhibit a wide host range both *in vitro* and *in vivo*; third, copious amounts of virus and viral products can be produced in cells in culture (Graham, 1990).

In the construction of recombinant adenoviruses, the foreign gene of interest is first inserted into a subgenomic viral fragment propagated in a bacterial plasmid. The resulting construct is then used to transfect mammalian cells with either a second viral DNA fragment or a second plasmid containing the remainder of the viral genome plus the overlapping sequences which are required for homologous recombination. Identification of the viral recombinants can be done by plaque isolation and restriction analysis (Berkner, 1988).

For the generation of helper independent recombinants, deletion of the E3 region of the virus can result in vectors with a capacity of 4-5 kb. However, when both E1 and E3 regions are deleted, such vectors can have the capacity of 7-8 kb but they are defective and need to be grown in 293 cells which express the E1A gene. Larger sizes of inserts can also create packaging constraints resulting in a reduced virus structural stability (Graham *et al.*, 1977; Graham, 1990).

Many adenovirus recombinants have been constructed expressing the HIV envelope gene (Dewar *et al.*, 1989), the HBV surface antigen (Morin *et al.*, 1987; the HSV-1 gE and gI genes (Johnson *et al.*, 1988; Hanke *et al.*, 1990), the pseudorabies gp150 gene (Eloit *et al.*, 1990), the VSV glycoprotein gene (Prevec *et al.*, 1989; Schneider *et al.*, 1989), the CMV gB gene (Marshall *et al.*, 1990) and the rabies glycoprotein gene (Prevec *et al*, 1990). Furthermore, all of the investigators have found properly processed recombinant proteins which maintain their functional and antigenic characteristics (Rosenfeld *et al.*, 1991). In addition, Thummel *et al* (1982) found that placing the heterologous gene under the control of the adenovirus major late promoter results in greater expression than when an external promoter is used.

A concern in the usage of adenovirus vectors is the size limitation of the inserts as compared to the other DNA virus vectors. For instance, a vaccinia virus vector can accommodate inserts up to four times larger than adenoviruses (Graham, 1990). A potential problem one might encounter in the use of adenovirus type 5 as a vaccine in the human population is that a large percentage

of people have already been exposed to the virus, thus creating a problem in stimulating a good immune response. In addition, adenoviruses are capable of oncogenic transformation in cell culture and in some rodents (Nevins, 1987). This has raised concerns as to the use of adenovirus-based vaccines. However, no direct association has been made between adenoviruses and human cancers and it seems highly unlikely that any will be. Nevertheless, adenovirus vaccines consisting of adenoviruses types 4 and 7 have been used by the military to immunize recruits against acute respiratory disease (ARD) since 1965 (Channock *et al.*, 1966; Chaloner-Larsson *et al.*, 1986; Couch *et al.*, 1963). No undesirable side effects have been observed and the vaccines have induced long lasting immunity (Edmondson *et al.*, 1966; Griffin *et al.*, 1970; Top *et al.*, 1971a; Top *et al.*, 1971b; Larson *et al.*, 1986)

3. STABILITY OF VIRAL VACCINES

3.1 Introduction

The production of vaccines has mostly centered around and been dependent on the cold chain from the time of preparation to the time of administration (Hilleman, 1989; Cheyne, 1989). This is mainly due to the inactivation of viruses at higher temperatures (Pickup *et al.*, 1991; Russell *et al.*, 1967; Lucasse and Visser, 1978; Budowsky, 1991). However, in the oral vaccination of wildlife, vaccine will be enclosed in baits and dropped in the field. Once on the ground, a vaccine will be exposed to environmental fluctuations. The success of any vaccination campaign depends on the vaccine remaining viable long enough

to immunize the target animals.

3.2 Improving viral stability

Some of the ways that have been used to improve vaccine stability are the use of osmotic stabilizers and the use of ionic compounds (Burfoot *et al.*, 1977; Frerich and Herbert, 1974; Hekker *et al.*, 1973). The use of osmotic stabilizers is probably the most common. They generally have very little effect on the virus but affect the bulk solution properties of water. Polyols and sugars (glycerol, mannitol, glucose, fructose, sucrose, etc) are used in concentrations of 10-40% to stabilize the lattice structure of water thus increasing the surface tension and viscosity. They also prevent viral aggregation by increasing the molecular density of the solution. Some polymers (dextran, levans, polyethylene glycol) at 1-15% concentration can also lower viral aggregation. Some ionic compounds (chloride, citrate, sulfates, acetate, quaternary amines) between 20-400 nM can stabilize viruses by shielding charges (Hilleman, 1989; Schein, 1990).

In addition to chemical stabilizers, other techniques can be employed to improve viral stability (Hilleman, 1989). These include:

a) Freezing, with maintenance of a constant temperature to prevent shifts in ice crystals.

b) Lyophilization, with an optimal residual moisture and with a matrix structure provided by an excipient such as gelatin or gelatin.

c) Purity, with freedom from destructive enzymes.

d) Freedom from natural or added surfactants.

e) Optimal pH.

f) Darkness and lack of photo-active substances.

Various studies have looked at the effects of environmental conditions on the survival of viruses (Parker *et al.*, 1944). For instance, most nonenveloped viruses are inactivated faster under relative humidity between 20-30% and enveloped viruses are more unstable at relative humidity levels of 70 to 80% (Hemmes *et al.*, 1960; Miller and Artienstein, 1967; Parker *et al.*, 1944; Davis *et al.*, 1971; Donaldson and Ferris 1976; Estes *et al.*, 1979; Sattar and Ijaz, 1987).

4. DELIVERY METHODS FOR ORAL VACCINES

4.1 Introduction

Oral immunization represents an attractive and feasible alternative over systemic immunization routes since oral vaccines are easy to administer, relatively free from side effects and allow for frequent boosting. However, as compared to systemic immunization, oral immunization requires a much larger dose of the viral antigen because the antigens may be destroyed in the stomach by gastrointestinal enzymes, pH and selective adsorption in the intestinal tract. Several methods are employed to overcome this inefficiency including the use of liposomes to incorporate the antigens, cholera toxin B subunit to conjugate the antigen, and microencapsulating the antigen (McGhee *et al.*, 1991; Hagen, 1990; Metha, 1986; McGhee and Mestecky, 1989). This study is concerned with the latter method and, therefore, this review is limited to the microencapsulation method of antigen delivery.

4.2 Microencapsulation

The use of microencapsulation as a delivery method for substances has been widely adopted in the pharmaceutical industry since the first patent in 1949. Many different materials have been encapsulated using various coating materials (Metha, 1986; Hagen, 1990; Lin and Kawashima, 1987). However, this study is concerned with two methods, the cellulose acetate phthalate (CAP) and sodium alginate methods.

The CAP method utilizes the solubility properties of CAP to create an enteric coating. This is due to the presence of an ionizable phthalate group; the polymer is insoluble when the pH is 2-5, but is soluble at pH >6. The polymer is relatively stable and has been used to microencapsulate both small molecular weight drugs (Merkle and Speiser, 1973; Alpar and Walters, 1981; Madan *et al.*, 1978) and viruses (Maharaj *et al.*, 1984). This method however does require the use of organic solvents for the formation of the microspheres and this can be detrimental to some viruses or their antigens (Bardell, 1975; Maharaj *et al.*, 1984).

In contrast, the sodium alginate method does not require any organic solvents and is used to microencapsulate mammalian cells which are found to remain viable within the microspheres (Bano *et al.*, 1991; Hagen, 1990). Sodium alginate, when added to a calcium citrate solution, results in the formation of a gel. A semi-permeable membrane can then be created by the addition of the beads to a solution of polyamino acids. This method however, is not suitable for enteric coating (Sinacore *et al.*, 1989; Bano *et al.*, 1991).

OBJECTIVES

The success of a field trial depends on the virus maintaining its infectivity to immunize the target species. Also, practical considerations for vaccine production require procedures which are simple and can be scaled up easily. Moreover, proper packaging of the virus for vaccination is just as crucial. The present vaccine (attenuated rabies virus) in the blister pack is not totally satisfactory and microencapsulation may be a suitable alternative.

This study therefore had three main objectives:

1). To determine the stability of the recombinant virus under different conditions:

a) Upon storage in a chemical stabilizer or in EBSS under refrigeration (4^0C) or at room temperature (23 ± 2^0C).

b) In relation to the thermostability of its parental virus (deleted E3 region) and a wild-type adenovirus type 5.

c) On lyophilization and storage at refrigeration and at room temperatures.

d) Under conditions of low (20±5%), ambient (45±5%) and high (80±5%) relative humidity when suspended in skunk or fox feces and placed on stainless steel disks.

e) Upon exposure to selected hard-surface disinfectants.

f) Upon field exposure in the baited vaccine in the Fall season.

2) To improve the recombinant virus titre using methods potentially suited for vaccine production.

3) To microencapsulate the recombinant as an alternative approach to vaccine packaging and delivery.

MATERIALS AND METHODS

1. CELLS

1.1 MRC-5

The MRC-5 human embryonic lung cell line was used for the virus production studies. The cells were initially obtained from Connaught Laboratories Limited (Willowdale, Ontario) and later from the Regional Virology Laboratory, Children's Hospital of Eastern Ontario, Ottawa. They were routinely grown in 75 cm^2 plastic flasks (Costar, Cambridge, MA) using the CMRL-1969 medium (Healy *et al*, 1971) (Connaught Laboratories) supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, NY) and 0.225% (w/v) sodium bicarbonate (BDH Chemicals, Toronto, Ontario).

For passaging of cell cultures, the monolayers were washed twice with Dulbecco's Ca^{++}- and Mg^{++}-free phosphate buffered saline (PBS; pH 7.2). Each monolayer then received approximately 2 mL of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; Gibco, product no. 610-5300AG) and the flasks were then rotated to spread the solution evenly. The trypsin-EDTA mixture was decanted immediately and the flasks placed in a 37°C, 5% CO_2 incubator for 3 minutes. Three mL of fresh growth medium were added to each flask and the cells were pipetted thoroughly. The cell suspension was then divided into three new flasks of the same size. Twenty mL of the growth medium were then added to each flask. Complete monolayers usually formed in four days when the flasks were incubated at 37°C.

1.2 Vero

The Vero line of African green monkey kidney cells was used throughout this study for virus titration. A seed culture of these cells was provided by Dr. J.B. Campbell of the University of Toronto (Toronto, Ontario). The cells were grown in Eagle's minimal essential medium (EMEM; Gibco) supplemented with 5% fetal bovine serum (Cell Culture Laboratories, Cleveland, OH), 50 µg/mL gentamicin sulfate (Cidomycin; Roussel, Montreal, Quebec), 200 mM glutamine (Gibco) and 0.225% (w/v) sodium bicarbonate (BDH). The cells were maintained in the same medium but with only 2% serum. They were passaged as described for MRC-5 cells.

2. VIRUSES

The adenovirus-rabies recombinant (rHAd5-RG1) virus (Prevec *et al.*, 1990) used throughout this study was in its third passage in MRC-5 cells and the pool used by us was prepared in Dr. Campbell's laboratory. We received the virus through the courtesy of Dr. A. Wandeler, Animal Diseases Research Institute (ADRI), Agriculture Canada, Nepean, Ontario.

The parental virus of rHAd5-RG1 consists of the human type 5 adenovirus with the E3 region deleted. This virus was kindly provided by Dr. L. Prevec of McMaster University (Hamilton, Ontario). The parental virus was grown in MRC-5 cells and concentrated by polyethylene glycol (PEG 800) hydroextraction before use.

The wild-type human adenovirus type 5 was kindly provided by Mr. R.

Raphael of the Bureau of Biologics, Health and Welfare Canada (Ottawa, Ontario).

2.1 Virus titration

2.1.1 Plaque assay

For the virus plaque assays, monolayers were prepared by seeding a 2.0 mL suspension of approximately $1X10^5$ Vero cells into each well of a 12-well cell culture plate (Costar, Cambridge, MA). The seeded plates were incubated at 37°C overnight in a 5% CO_2 incubator. Serial tenfold dilutions (10^{-3}, 10^{-4}, 10^{-5}) of the virus samples to be titrated were made in Earle's balanced salt solution (EBSS; Gibco). For each dilution, 3 wells were inoculated with 0.1 mL of the inoculum each. The cell control wells were exposed to an equivalent amount of EBSS. The plates were rotated gently to redistribute the inoculum over the monolayers before incubating them at 37°C for 60 min in a 5% CO_2 atmosphere. Redistribution of the inoculum was carried out every 10 min during the virus adsorption period.

At the end of the adsorption period, each well received 2.0 mL of an overlay containing EMEM with 200 mM glutamine (Gibco), 10 mM magnesium chloride (Sigma Chemical, St Louis, MO), 50 µg/mL gentamicin, 2 µg/mL amphotericin B (Fungizone; Gibco), 2% serum, 0.225% (w/v) sodium bicarbonate and 0.75% (w/v) agarose type II (Sigma). The plates were kept at room temperature to allow the medium to solidify, sealed individually in laminated plastic bags (Dazey Corp., Industrial Airport, KS) and incubated at 37°C, for 5-6 days. Once plaques were readily visible under an inverted microscope, 2.0 mL of a 10% Formalin (Fisher Scientific, Ottawa, Ontario) solution in normal saline was added to each

well to fix the monolayers and to inactivate the virus. After incubating the plates overnight at room temperature, the overlay was removed with running tap water. Each monolayer was then stained for 5 min with 0.5 mL of a 1% aqueous solution of crystal violet. The stain was washed off in tap water, the plates dried and the plaques counted. The virus infectivity titre is expressed as plaque forming units (PFU) per mL.

3. VACCINE

3.1 Production of vaccine

3.1.1 Roller bottles

For the production of the recombinant virus corrugated roller bottles (Corning Glass Inc, Corning, NY) with approximately 1700 cm^2 surface area were used. Twenty-five mL of MRC-5 cell suspension containing approximately $1X10^5$ cells/mL was placed into each roller bottle and gassed with CO_2 to maintain neutral pH. The bottles were rolled at 0.25 rpm for 6 hr to allow the cells to adhere to the plastic surface. An additional 75 mL of CMRL-1969 growth medium was added to each roller bottle and the rotation speed increased to 0.5 rpm in a 37^0C incubator. The bottles were monitored daily for monolayer formation.

Once a monolayer was formed, the growth medium was decanted and the roller bottles washed with EBSS to remove the residual medium. The bottles were then infected with the recombinant virus and incubated for 1 hr. Fifty mL of maintenance medium were then added and the bottles incubated until cytopathology was evident in 75% of the monolayer.

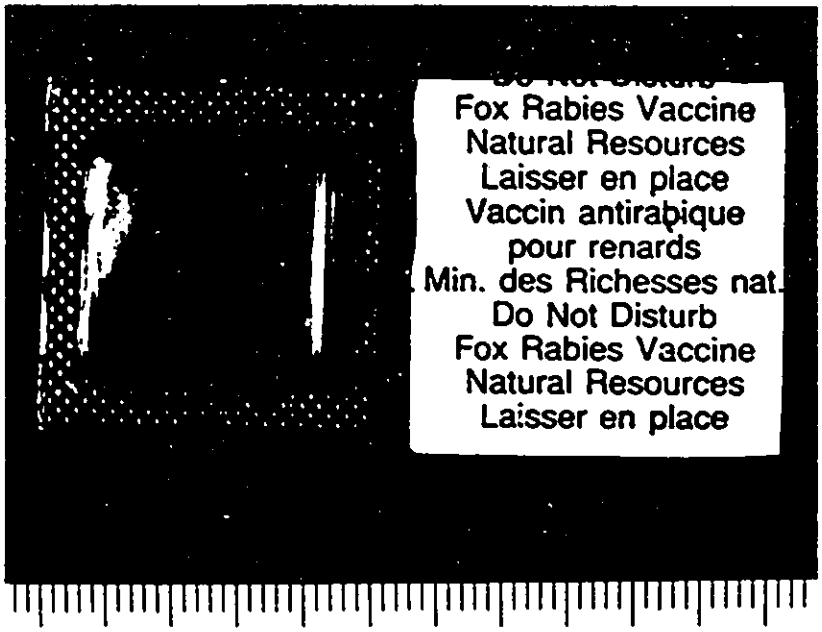
3.2 Vaccine for field trials

3.2.1 Preparation of baited vaccine

Polystyrene blister packs and a bait mixture were kindly provided to us by Dr. K. Lawson of Connaught Laboratories and both of these are currently being used in the field trials of the attenuated (ERA) rabies virus vaccine (Figure 1)(Bachman *et al.*, 1990). One part of the stock virus was mixed with one part of EBSS or one part of the stabilizer under test and 2.0 mL of this mixture were injected into each blister pack using a 2.0 mL syringe with a 26-gauge needle (Becton, Dickinson & Co., Mississauga, Ontario). The needle puncture was then sealed with a plastic glue. For embedding the blister packs in the bait, the bait mixture, contained in a 250 mL glass conical flask, was melted in a 70^{0}C water bath. Approximately 18 gm of it were then poured into each one of the clear plastic moulds (4.0 X 4.0 X 2.5 cm). While the bait mixture was still soft, the blister pack with the virus was inserted into it with an applicator stick making sure that the entire surface of the blister pack was completely and evenly covered with the bait (Figure 2). To monitor the vaccine temperature, 16-gauge hypodermic probes (Product No. Hyp3-16-1 1/2-K-V-48RP; Omega Corp., Stamford, CT) containing type K thermocouples were inserted into two of the blister packs before embedding them in the bait. The bait temperature was recorded by plugging the thermocouple probes into a portable thermometer (Omega).

Figure 1. The empty blister pack (scale in mm).

Figure 2. Blister pack and bait-coated vaccine (scale in mm).


Fox Rabies Vaccine
Natural Resources
Laisser en place
Vaccin antirabique
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Do Not Disturb
Fox Rabies Vaccine
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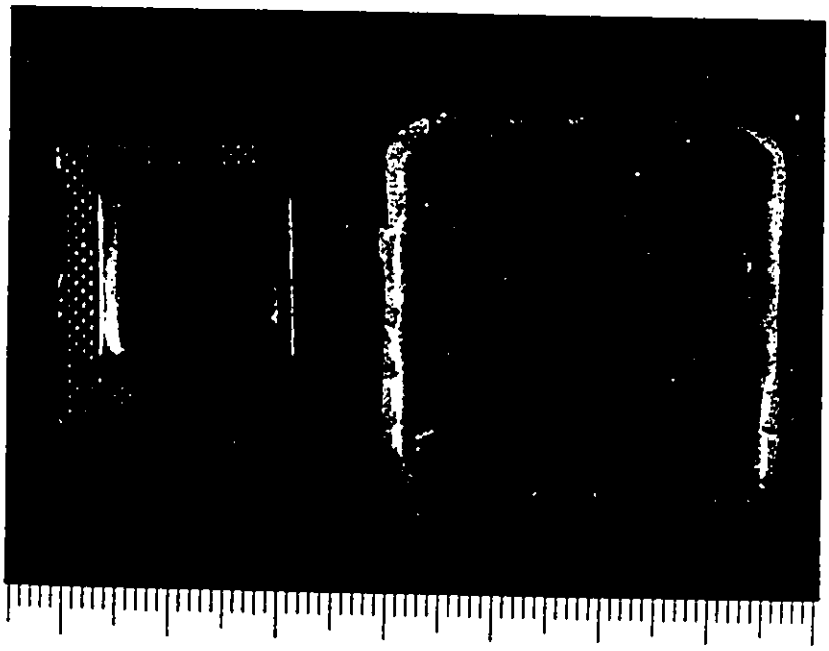


4. INDOOR SURVIVAL EXPERIMENTS

4.1 Thermostability of rHAd5-RG1 in suspension

The stock virus was mixed in a 1:1 ratio with an egg yolk-containing commercial stabilizer provided by Dr. Lawson. This stabilizer is used with the attenuated (ERA) rabies virus vaccine. The virus-stabilizer mixture was vortexed for 10 s and 200 µL quantities were aliquoted into 2.0 mL sterile plastic vials (Sarstedt, St. Laurent, Quebec). As virus control, the stock virus was mixed with an equal volume of EBSS. Immediately, 100 µL samples of the virus-EBSS and the virus-stabilizer mixtures were removed for titration (zero min controls).

Five vials of each of the test mixtures were placed at room temperature (23±2°C) in a plastic box; a corresponding set was placed at 4°C. One vial of each mixture was removed at the end of 2, 4, 8, 12, and 16 days. These samples were stored at -80°C and all of them were titrated at the same time at the end of the experiment. This experiment was repeated three times.

4.2 Comparative stability of rHAd5-RG1, its parental virus and a wildtype human adenovirus type 5

To determine the effect of the genetic insertion on virus thermostability, we compared the recombinant virus, its parental virus (deleted E3 region) and a wildtype human adenovirus type 5. Vials with 0.1 mL aliquots of the three viruses were then kept in a 37°C incubator and vials were removed on days 0, 1, 2, 4, 8, 16, 32, and 40. At the end of the experiment, all the samples were titrated by plaque assay at the same time.

4.3 Thermostability of lyophilized rHAd5-RG1

Aliquots (0.1 mL) of the stock suspension of rHAd5-RG1 were added to sterile 1.5 mL tubes; an aliquot was added to 0.9 mL EBSS to determine the initial virus titre. The remaining tubes were lyophilized in a freeze drying chamber (Virtis Company, Gardiner, NY). Immediately after the freeze drying process, 0.9 mL of sterile distilled water was added to a tube as the zero time sample. The remaining tubes were placed either at 4^0C or at room temperature and sampled at 1, 2, 4, 8, 16, 32 and 40 days. The material in the tubes was reconstituted with sterile distilled water and titrated by plaque assay.

4.4 Stability of rHAd5-RG1 under different relative humidity settings

To determine the survival of the recombinant virus at different relative humidity (RH) settings on non-porous inanimate surfaces, it was suspended in fecal samples from an unvaccinated fox and an unvaccinated skunk kept at the ADRI. The fecal samples were prepared as a 10% (w/v) suspension in normal saline, clarified by centrifugation at 1,000 xg for 5 min and passed through a 47 mm diameter membrane filter (Nalge Co, Rochester, NY) with a pore diameter of 0.22 µm to remove bacterial and fungal contamination. The filtrates were found to be non-cytotoxic to Vero cells and free from any indigenous viruses that could produce cytopathology in these cells.

A 1:10 dilution of the virus was prepared in either EBSS or in the fecal filtrates and, using a positive displacement pipette (Gilson, Villiers le Bel, France), 10 µL of virus suspension were placed separately at the centre of stainless steel

disks (1 cm in diameter). The disks were punched out of locally purchased sheets (0.75 mm thick) of #4 polished stainless steel. A 12-well plastic cell culture plate, with one virus-inoculated disk per well, was held uncovered in a laminar flow hood (with the blower turned off) under ambient conditions. The air temperature and relative humidity were monitored throughout the experiment using a recording hygrothermograph (Cole-Parmer, Chicago, IL). One disk of each of the two different virus suspensions was removed at the end of 1, 2, 4, 8, 24, 48, and 72 hr and placed in a tube containing 1.0 mL EBSS. The tubes with the disks were sonicated for 10 min in an ultrasonic bath (Bransonic Ultrasonic Corp, Danbury, CT) to assist in the elution of the virus from the disks. The eluates were then titrated. This experiment was performed twice with fox feces and once with skunk feces.

For the experiments on the effects of high relative humidity (75-85%) on virus survival, air entering the constant temperature (20°C) incubator was bubbled through sterile deionized water. The low level of RH (15-25%) was obtained by passing the air through a column of Drierite (Hammond, Xenia, OH). In both cases the air temperature and RH were continuously monitored.

5. OUTDOOR SURVIVAL EXPERIMENTS

5.1 Baited vaccine field exposure

Baits with the attenuated rabies virus vaccine are currently dropped in the fall season in Eastern Ontario. Therefore, the survival of rHAd5-RG1 in baits under outdoor conditions was also tested at this time (October) of the year.

Each blister pack containing 2.0 mL of the recombinant virus (grown in MRC-5 cells) was embedded in the bait. The baits were placed outdoors, with one set of samples exposed to sunlight and another placed in the shade; a thermocouple-fitted vaccine pack was included in each set to measure bait temperature. Outdoor air temperature and relative humidity were also monitored with a recording hygrothermograph throughout the sampling period. Samples were withdrawn at 1, 2, 4, 8, 16, and 32 days and titrated.

A set of samples containing the following organic materials was also prepared in order to determine their virus stabilizing potential; peptone (Oxoid Ltd., Basingstoke, Hampshire, England), lactalbumin hydrolysate (Gibco), yeast extract (Difco Laboratories, Detroit, MI) and tryptose phosphate broth (Difco). All of the above were prepared as 10% (w/v) solutions in phosphate buffered saline (pH 7.2) and each was mixed with the virus in a 1:1 ratio. The mixtures were placed in blister packs and inserted into baits as described above. After 32 days of exposure in a sun-exposed area, the contents were titrated.

6. VIRUS CONCENTRATION

6.1 Hydroextraction

In an effort to increase the viral titre using technically simple methods, we looked at hydroextraction using polyethylene glycol (PEG P-8000; Sigma) and polyacrylamide (Aquacide IV; Calbiochem, San Diego, CA).

In the PEG method, an appropriate length (0.25 m) of dialysis tubing (2.5 cm diameter) was autoclaved in a beaker containing double distilled water. Upon

cooling, the bag was knotted at one end and 50 mL of the viral suspension was added to the bag. The other end of the bag was also sealed by knotting. The dialysis bag was then immersed in a container with PEG making sure the bag was completely covered with the powder. The container was placed at 4°C overnight (Ramia and Sattar, 1979).

The bag was removed from the container and its outside washed thoroughly in running tap water. The remaining viral suspension was removed with a pipette and placed in a tube. The volume was reconstituted to 5 mL with PBS.

With Aquacide IV, 9 grams of it were added to a container with 50 mL viral suspension and incubated for 5 hr at 4°C for equilibration. The remaining viral suspension (about 5 mL) was pipetted into a tube. Both viral suspensions, before and after the hydroextraction, were titrated by plaque assay.

6.2 rHAd-RG1 concentration by detergents, Freon and lysis buffers

In an effort to produce a high titre of the recombinant virus using methods that are applicable for scaling up the production, we looked at mild detergents, Freon and lysis buffers as ways to release the cell associated virus particles. The virus was cultivated in MRC-5 cells as previously described. Aliquots of 10 mL of the virus infected cell suspensions were added to sterile tubes and NP-40 (Nonidet P-40; Sigma), Triton X-100 (J.T. Baker Chemicals Co., Phillipsburg, NJ), CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; Sigma), and sodium deoxycholate (Sigma) were added separately at a final concentration

of 0.1%. Also an equal part of Freon (1,1,2-trichloro-1,2,2 trifluoroethane; Photrex, J.T. Baker C., Phillipsburg, NJ) was added to a tube and another tube was left untreated to act as the control. The tubes were incubated at 4^0C for 30 min and vortexed for 2 min. All the samples were titrated by plaque assay.

To determine the effects of lysis buffers on improving the viral titre, buffer A (20 mM magnesium chloride, 1.5% NP-40) and B (1% NP-40, 1% Triton X100, 50 mM magnesium chloride, 50 mM tris hydrochloride) were used at a final concentration to 10 mL of cell-associated virus suspension and incubated at 4^0C for 30 min and vortexed for 2 min. All the samples were titrated before and after the buffer treatment.

7. MICROENCAPSULATION OF BSA AND OF rHAd5-RG1

7.1 Isotopic labelling of rHAd5-RG1

A monolayer of MRC-5 cells was prepared in a 75 cm^2 flask. rHAd5-RG1 was used to infect the flask with an MOI of 5 for 1 hr. The medium was removed and the cells were washed twice with EBSS. A 10 mL volume of medium without methionine (Flow laboratories, Irvine, Scotland) was added to the cells and incubated for 5 hrs. Radiolabelled ^{35}S-methionine (50 µCi/mL) (NEN Research Products, Wilmington, DE) was added to the medium and the flask was incubated for 48 hrs. The medium was then removed and the cells washed with EBSS twice, and 10 mL of CMRL-1969 medium was added to the cells. The flask was placed at -20^0C and it underwent 3 cycles of freezing and thawing to facilitate the release of the radiolabelled virus. The isotope-labelled virus

suspension was placed in a dialysis bag, knotted and immersed in a beaker containing 250 mL PBS. The beaker was then incubated at 4°C overnight. This procedure was repeated twice to remove the free isotope. The virus suspension was removed and stored at -80°C until further usage.

7.2 Cellulose acetate phthalate microencapsulation

The core material was prepared by mixing 5 mL of the radiolabelled virus with 5 mL of 5% sucrose and 5 mL of 5% cornstarch. The mixture was stirred at 50 rpm for 5 min and lyophilized. One hundred milligrams of the lyophilized powder was suspended in 200 mL of white paraffin oil and stirred at 206 rpm for 5 min. To the suspension, 20 mL of 10% (w/v) cellulose acetate phthalate (ICN Biomedicals Inc, Cleveland, OH) in acetone-95% ethanol (9:1) was added. The stirring was continued for 5 min to allow the microspheres to form. Once the microspheres were formed, 75 mL of chloroform (Sigma) were added. The suspending medium and chloroform were decanted, and the microspheres were washed briefly in another 75 mL chloroform and then air-dried at room temperature and stored at 4°C (Fig. 3).

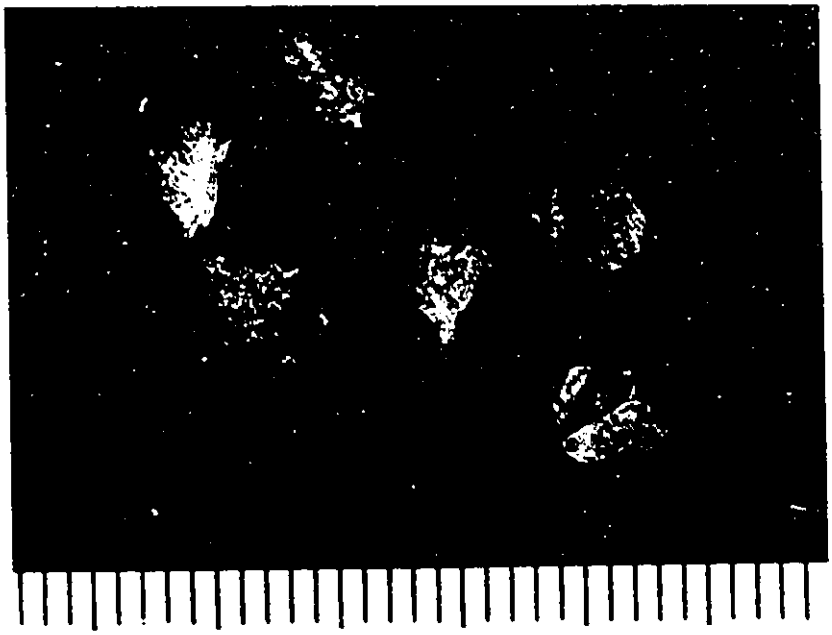
In one set of experiments, diethyl phthalate (DP; BDH, Toronto, Ont.) was added to increase the pliability of the microcapsules. DP was added to the cellulose acetate phthalate solution to a final concentration of 3% before adding it to the paraffin oil.

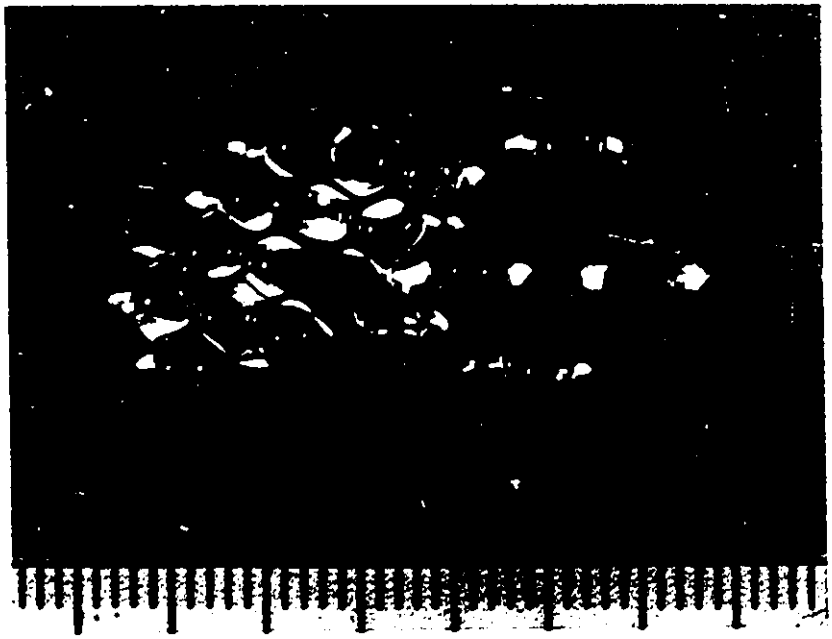
7.3. Sodium alginate microencapsulation

One milliliter of radiolabelled virus suspension was added to 3 mL 1.2%

Figure 3. Cellulose acetate phthalate microcapsules (scale in mm).

Figure 4. Sodium alginate microcapsules (scale in mm).





(w/v) sodium alginate in PBS and stirred at 50 rpm for 3 mins.

The microcapsules were formed by adding the suspension into a 1 mL pipette inserted into a Pasteur pipette and letting 0.1 mL droplets be released into a 500 mL beaker containing 100 mL 1.3% calcium carbonate solution. The gelled droplets were kept in suspension for 3 min, then allowed to settle and the supernatant was removed by decanting and stored at 4^0C (Fig. 4).

7.4 Release of the core material

Samples of bovine serum albumin (BSA; Sigma) were encapsulated by the CAP method as described above and the release of the core material was determined by incubating 0.1 gm of the microcapsules in 5 mL saline at pH values of 1.5, 3.0, 4.5, 6.0, 7.5, and 9.0 at 37^0C for 4 hr. Samples of 1 mL were removed every hour, and the protein content was determined spectrophotometrically at 280 nm.

The release of the virus was monitored by the release of the ^{35}S-radiolabelled virus into simulated gastric and intestinal juices. Approximately 0.1 gm of the microencapsulated (sodium alginate, cellulose acetate phthalate) material was added to 20 mL of simulated gastric juices USP (0.08 M HCL containing 0.2% NaCl, pH 1.2) without pepsin. The suspension was incubated for 3 hr at 37^0C with shaking at 100 rpm. Samples of 1 mL were removed every hour, clarified by microfuging for 1 min and an aliquot of 0.1 mL was added to a scintillation vial containing 0.4 mL of Aquasol scintillation fluid (NEN Research Products; Wilmington, DE). The vial was shaken and counted in a scintillation

counter (LKB Products; LKB Wallac Oy, Turku, Finland).

After the 3 hour period, the simulated gastric fluid was decanted and the remaining capsules were washed twice with double distilled water and resuspended in 20 mL of simulated intestinal juice (0.05 M KH_2PO_2 adjusted to pH 7.5 with 0.04 M NaOH) without pancreatin. Samples were taken and counted every hour as previously described. The pH was monitored every 30 min and adjusted as necessary with 1M NaOH.

8. CHEMICAL DISINFECTION OF rHAd5-RG1

To study the comparative efficacy of disinfectants on the recombinant virus, we selected those that are commonly used for the decontamination of environmental surfaces and medical instruments. Products included were 1% domestic bleach (Javex; Colgate Palmolive, Toronto, Ontario), 2% glutaraldehyde (Cidex; Surgikos Canada Inc., Peterborough, Ontario), 70% ethanol (Sigma Chemicals) and Lysol Disinfectant Spray (L & F Products, Montvale, NJ). The disinfectants were tested on virus-contaminated non-porous surfaces.

The recombinant virus was suspended in 5% (v/v) fetal bovine serum (Gibco) to act as the organic load. Using a positive displacement pipette, 10 µL of the virus were placed at the centre of each of several stainless steel disks. One disk was removed immediately and placed in a vial with 990 µL EBSS. The remaining disks with the virus inoculum were allowed to dry for 1 hr under ambient conditions (45±5% RH; 23±2°C). A 10 µL aliquot of the virus suspension was also added directly to 990 µL EBSS to serve as input virus control. After the

drying of the virus inoculum, one disk was removed and added to a vial with 1 mL of EBSS. Twenty microliters of the disinfectant under test were added to the dried inoculum on each of the remaining disks and incubated for 10 minutes at room temperature. Each of the disks was then added to a glass vial with 980 µL EBSS. The vials were sonicated in a water bath (Bransonic) for 10 min and the samples were titrated by plaque assay (Sattar *et al*, 1989).

9. DATA HANDLING AND STATISTICS

Each experiment was conducted at least twice and two sets of samples were normally used in each experiment; each sample was then titrated in duplicate. The data derived were plotted with the means and standard deviations calculated by the SigmaPlot™ software (Jandel Scientific, Corte Madera, CA).

For the calculation of the half-lives, a regression analysis was used to derive the constant (K_i) and the correlation coefficient. This was also done using SigmaPlot. The half life ($t½$) was then calculated by the formula $t½ = 0.693/K_i$. A standardized t-test (paired) was used to test for significance between two variables using an alpha value of 0.05 and a P value was calculated using SigmaPlot.

EXPERIMENTS AND RESULTS

1. VIRUS TITRATION BY PLAQUE ASSAY

All of the experiments conducted involved assaying for virus infectivity. It was, therefore, deemed necessary to establish a consistent viral assay system. In view of this, six cell lines (Vero, HEp2, HeLa, CV-1, 293, MRC-5) were screened for their ability to give readily obvious cytopathic effects (CPE) when infected with the recombinant virus.

Although HeLa, 293, and MRC-5 cells gave demonstrable CPE, the monolayer often peeled off from the surface of the culture plates when used in plaque assays. Vero cells gave the most consistent plaques that were readily visible by the 7th day. Initially, the plaques were generally small (0.3-0.5 mm) but the addition of 10 mM $MgCl_2$ in the overlay was found to expedite plaque formation from 7 to 5 days and the resulting plaques were slightly larger (0.5-0.6 mm) in diameter. Vero cells were also used for plaque assays for the parental as well as the wild-type adenovirus and their plaques were similar in size and appearance to those of the recombinant virus.

2. SURVIVAL OF rHAd5-RG1 UNDER INDOOR CONDITIONS

2.1 Influence of the Connaught Laboratories stabilizer on virus infectivity

It is important to protect the infectivity of a live vaccine virus during its storage. Apart from the use of lower temperatures, various chemical agents are incorporated in the vaccine to stabilize virus infectivity (Burfoot *et al.*, 1977). The ERA rabies vaccine currently being used in Ontario for wildlife vaccination

contains a chemical stabilizer which consists of a mixture of egg yolk, gelatin and Tris-HCl (Lawson *et al.*, 1988). These components are mixed in a ratio of 2:2:6, respectively. The effect of this stabilizer on the infectivity of the recombinant virus was tested.

The virus was suspended separately in EBSS (control), the three main components of the stabilizer as well as the whole stabilizer. These mixtures were kept under ambient conditions for 1 hr and then titrated. As can be seen in Figure 5, the virus suspended in gelatin gave the lowest titre, while that in Tris-HCl gave the highest titre in relation to the EBSS control; the virus suspended in the egg yolk or the whole stabilizer gave titres which were lower than the EBSS control. This indicates that the stabilizer exerts a slight inhibitory effect on virus infectivity or on its ability to produce plaques.

2.2 Storage of rHAd5-RG1 suspensions under indoor conditions

To determine the shelf-life of rHAd5-RG1, it was mixed with the whole Connaught Laboratories stabilizer or EBSS in a 1:1 ratio and stored at either room temperature (23±2°C) or refrigerator temperature (4°C). As can be seen from Fig. 6 and 7 and Table 3, the estimated half-lives of the virus in stabilizer solution held at 4°C and at 23±2°C were 88 and 64 days, respectively. The rate of loss of the virus infectivity in EBSS was somewhat greater, with the estimated half-lives at 4°C and at 23±2°C being 66 and 51 days, respectively. The results indicate that the virus is sufficiently stable in EBSS alone and for the remainder of the survival studies, EBSS without the stabilizer was used as the suspending medium.

Figure 5. Effects of the whole Connaught Laboratories stabilizer and its components on rHAd5-RG1 infectivity.

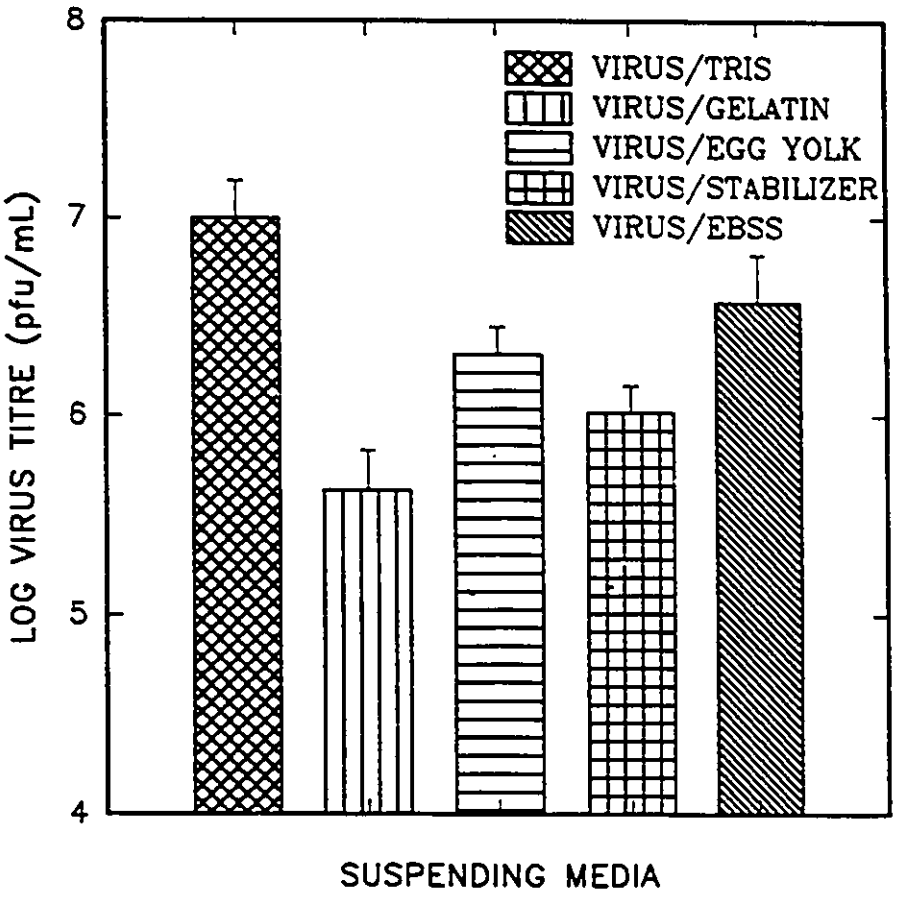

8
7
6
5
4
LOG VIRUS TITRE (pfu/mL)
VIRUS/TRIS
VIRUS/GELATIN
VIRUS/EGG YOLK
VIRUS/STABILIZER
VIRUS/EBSS
SUSPENDING MEDIA

Figure 6. Comparison of the stability of rHAd5-RG1 in the Connaught Laboratories stabilizer and in EBSS at room temperature.

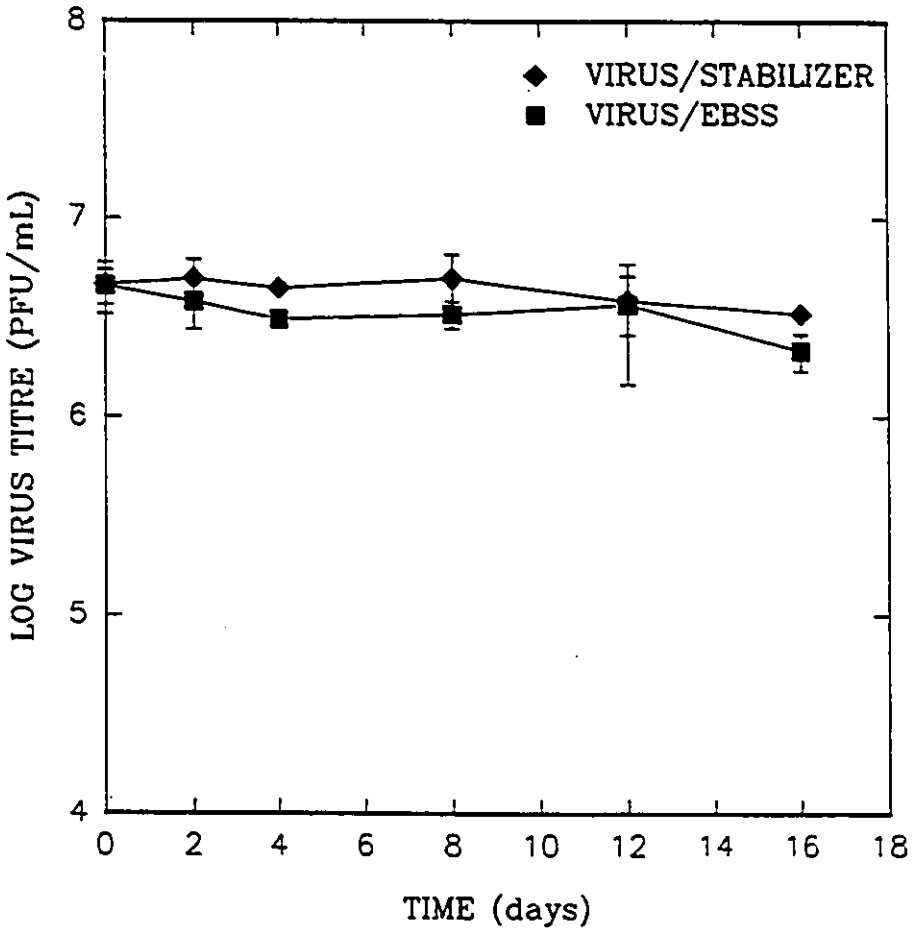

VIRUS/STABILIZER
VIRUS/EBSS
LOG VIRUS TITRE (PFU/mL)
TIME (days)
8
7
6
5
4
0
2
4
6
8
10
12
14
16
18

Figure 7. Comparison of the stability of rHAd5-RG1 in the Connaught Laboratories stabilizer and in EBSS at 4°C.

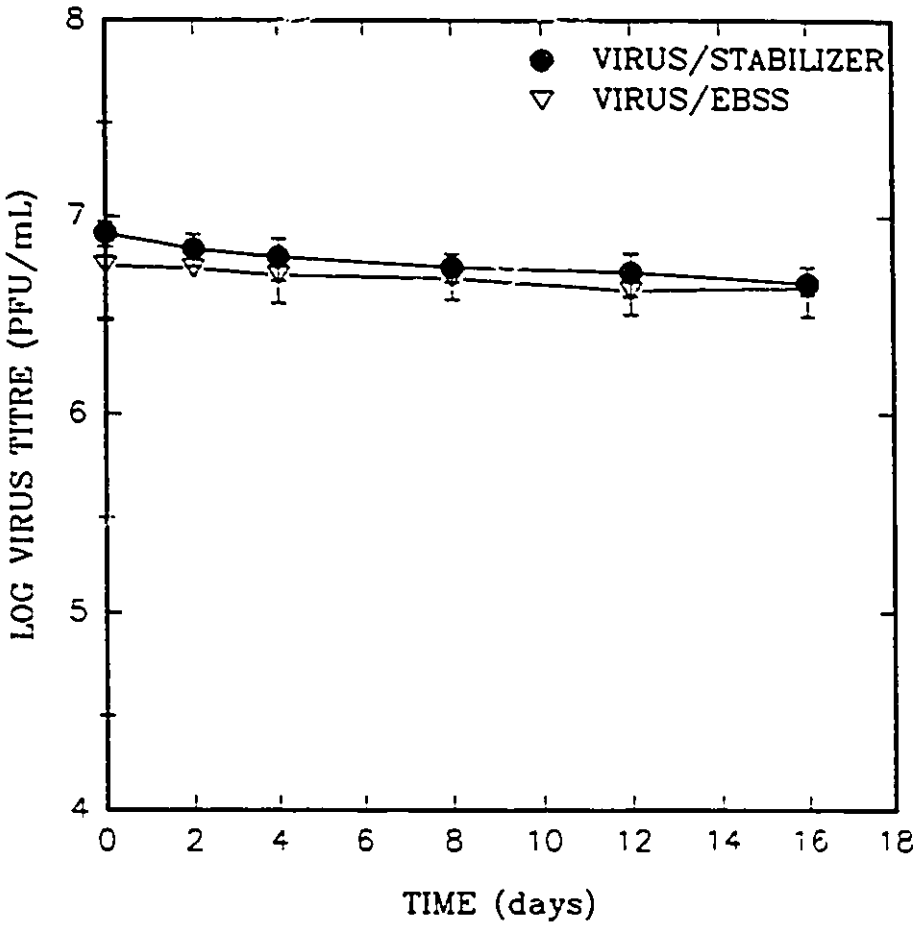

VIRUS/STABILIZER
VIRUS/EBSS
LOG VIRUS TITRE (PFU/mL)
TIME (days)
8
7
6
5
4
0
2
4
6
8
10
12
14
16
18

Table 3. Estimated half-lives and K_i values of rHAd5-RG1 on storage in suspension

EXPERIMENT	K_i[a]	CORRELATION COEFFICIENT[b]	HALF-LIVES (DAYS)
Stabilizer:			
4°C	0.0078	0.90	88
23±2°C	0.0106	0.83	64
EBSS			
4°C	0.0104	0.86	66
23±2°C	0.0135	0.84	51

[a]K_i = loss in $\log_{10}$ virus PFU/day
[b]Correlation coefficient refers to the measure of the linear association between two variables

2.3 Comparative stability of rHAd5-RG1, its parental virus and a wild-type human adenovirus type 5

To determine if the insertion of the ERA glycoprotein gene into the human adenovirus type 5 had altered the thermostability of the virus, we compared the recombinant with its parent virus (the adenovirus with a deleted E3 region) and a wild-type human adenovirus type 5. The three viruses were suspended in EBSS and held at 37°C to simulate an accelerated degradation (Jerne and Perry, 1954). The results show that the three viruses had similar survival kinetics (recombinant parental P = 0.52; recombinant and wild-type P = 0.38)(Fig. 8). In addition, the calculated half-lives in this test were 7.3, 10.0 and 6.2 days for the recombinant, parental and wild-type viruses, respectively (Table 4). Therefore, these data suggest that the genetic manipulations have not increased or decreased the thermostability of the recombinant virus when compared to the other two viruses.

2.4 Stability of rHAd5-RG1 in a lyophilized state

Certain types of viruses, for example polioviruses, cannot withstand lyophilization (Kovacs, 1964), whereas other types of viruses can not only be lyophilized but stored for extended periods in a lyophilized state. We therefore tested the ability of the recombinant to survive the process of lyophilization. The virus was suspended in EBSS, lyophilized and stored at either room temperature (23±2°C) or at 4°C. The results of these experiments are summarized in Fig. 9. After 40 days of storage, the lyophilized virus had lost less than a half-$\log_{10}$ in its

Figure 8. Comparative stability of rHAd5-RG1, its parental virus (deleted E3 region) and a wild type human adenovirus at 37°C in EBSS.

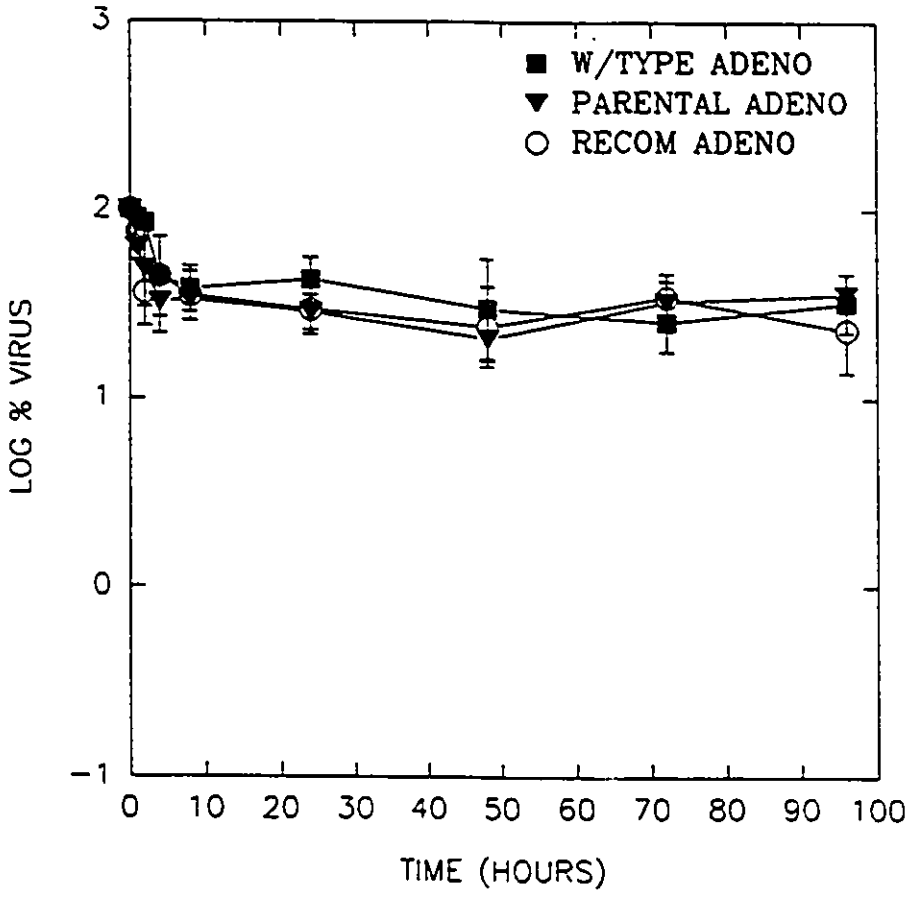
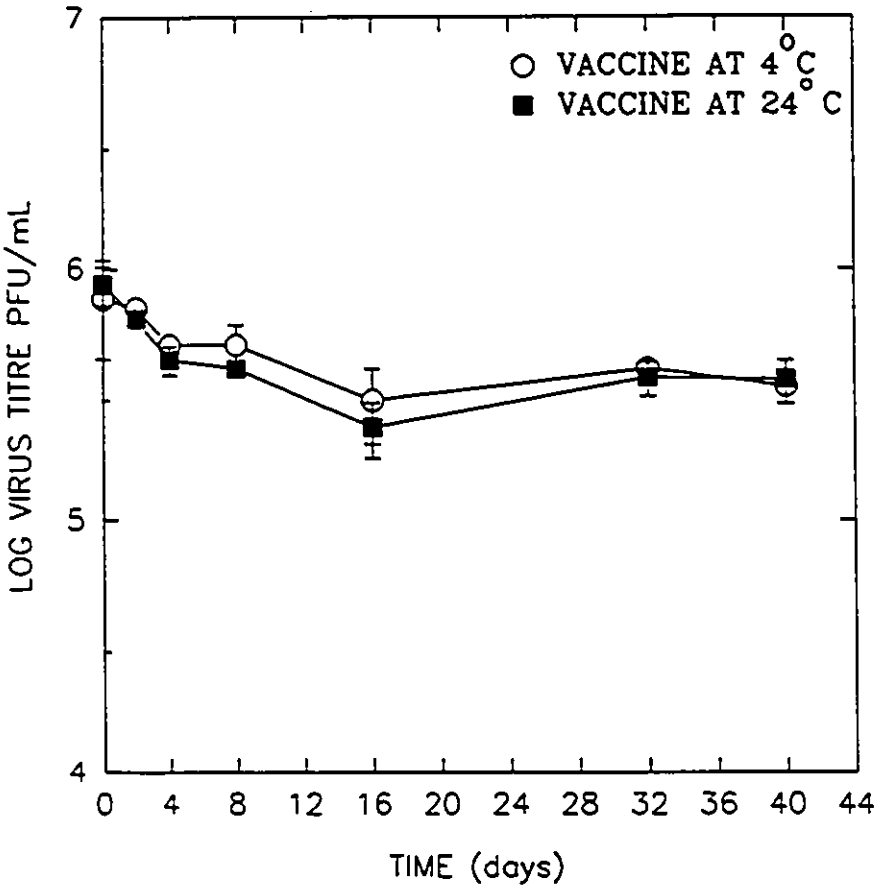

W/TYPE ADENO
PARENTAL ADENO
RECOM ADENO
LOG % VIRUS
3
2
1
0
−1
0
10
20
30
40
50
60
70
80
90
100
TIME (HOURS)

Table 4. Estimated half-lives and K_i values of rHAd5-RG1, its parental virus and a wild-type adenovirus type 5 when stored in EBSS at 37°C.

VIRUS	K_i	CORRELATION COEFFICIENT	HALF-LIVES (DAYS)
rHAd5-RG1	0.0040	0.65	7.3
parental virus	0.0027	0.50	10.0
wildtype virus	0.0047	0.74	6.2

Figure 9. Survival of the rHAd5-RG1 vaccine in a lyophilized state.


VACCINE AT 4°C
VACCINE AT 24°C
LOG VIRUS TITRE PFU/mL
7
6
5
4
0
4
8
12
16
20
24
28
32
36
40
44
TIME (days)

infectivity irrespective of the storage temperature (P= 0.495).

3. SURVIVAL OF rHAd5-RG1 ON ENVIRONMENTAL SURFACES

Excretion of the recombinant virus in the feces of vaccinated animals has been demonstrated (Charlton *et al*, 1992) and such virus discharge can lead to the contamination of environmental surfaces, including those in animal care facilities. Extended virus survival on such surfaces increases the likelihood of infection spreading to other animals or personnel involved in animal care. Virus survival on environmental surfaces indoors is influenced by RH (Sattar *et al.*, 1984; Mbithi *et al.*, 1991). The following experiments were conducted to examine the effects of various levels of RH on the survival of the recombinant.

The virus was suspended in either 10% feces (skunk or fox) or in EBSS and placed on stainless steel metal disks. The disks were kept at either low (20±5%), medium (45±5%) or high (80±5%) RH levels and virus survival tested over a period of 72 hr.

At the time these experiments were conducted the ambient RH was 45±5% and the air temperature 23±2^{0}C. Under these conditions, the calculated half-lives were 5.0 and 1.6 days for the virus when suspended in EBSS and in feces, respectively (Table 5). The most rapid loss of virus infectivity occurred within the first 8 hr after virus deposition, but the virus survived better in EBSS than in feces (Fig. 10).

At the low RH level, the half-lives of the virus were 2.4 and 3.8 days when it was suspended in feces and in EBSS, respectively (Fig. 11; Table 5).

At the high RH level, the drop in virus infectivity was more rapid in EBSS than in 10% feces (Fig. 12). This is in contrast to the results obtained at the medium RH. While the decline was continuous for the virus stored in EBSS, there was only an initial drop in viral titre when the virus was stored in feces. The calculated half-lives were 5.3 and 1.1 days for the virus kept in feces and EBSS, respectively (Table 5).

4. COMPARATIVE EFFICACY OF HARD-SURFACE DISINFECTANTS

Chemical disinfection of environmental surfaces is regularly carried out in animal care facilities as well as in laboratories handling infectious agents (Springthorpe and Sattar, 1990). Since the recombinant virus has been shown to retain its infectivity on environmental surfaces for several days under indoor conditions, it was considered important to determine which of the commonly used surface disinfectants are effective for decontamination.

The virus was suspended in 5% fetal bovine serum to simulate an organic load and placed on stainless steel disks. The inoculum was allowed to dry for 1 hr under ambient conditions and the disinfectant under test was placed on the dried inoculum and allowed to remain in contact with it for 10 min at room temperature. The virus was eluted from the control and from the disinfectant-treated disks and the eluates plaque assayed.

The results demonstrate that 70% ethanol, 2% glutaraldehyde, 1% domestic bleach, and Lysol Disinfectant Spray (contains 80% ethanol) could effectively inactivate the virus (Table 6). Thus, any of the tested disinfectants could be used

Figure 10. Survival of rHAd5-RG1 on stainless steel disks under ambient conditions (temperature 23±2°C, RH 45±5%). The virus was suspended in either EBSS or 10% fox feces.

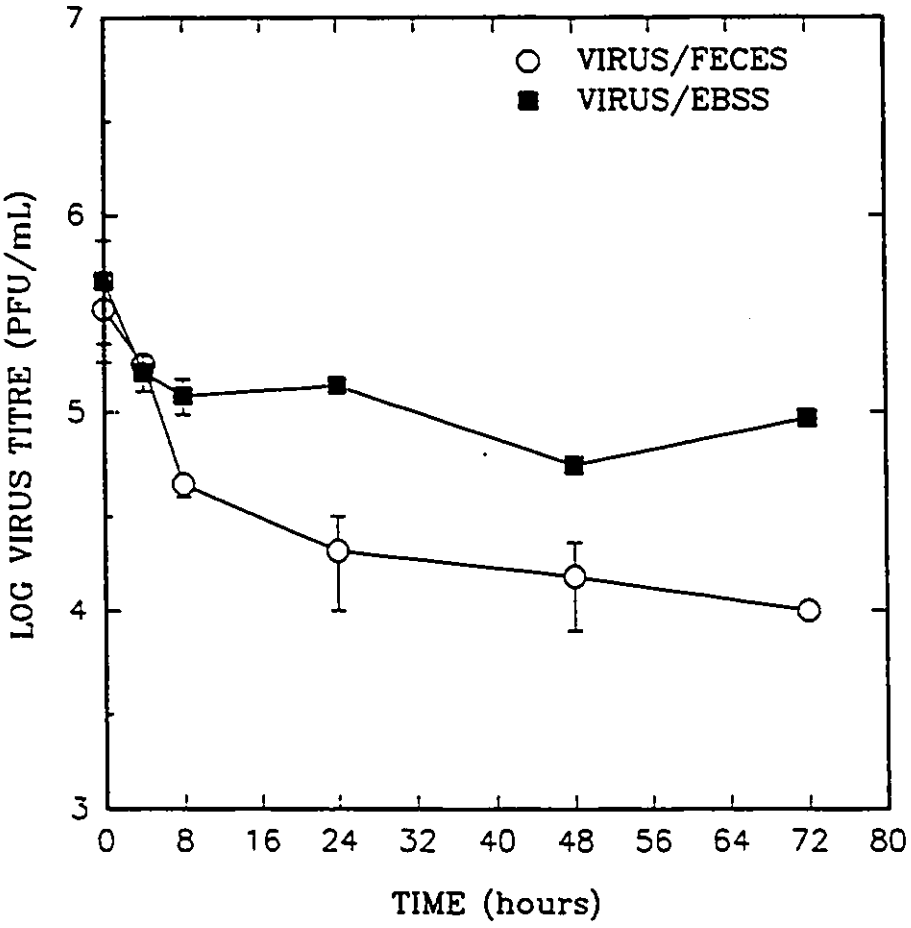

VIRUS/FECES
VIRUS/EBSS
LOG VIRUS TITRE (PFU/mL)
7
6
5
4
3
0
8
16
24
32
40
48
56
64
72
80
TIME (hours)

Figure 11. Survival of rHAd-RG1 on stainless steel disks under low RH (20±5%) and at ambient temperature (23±2^{0}C). The virus was suspended in either EBSS or 10% fox feces.

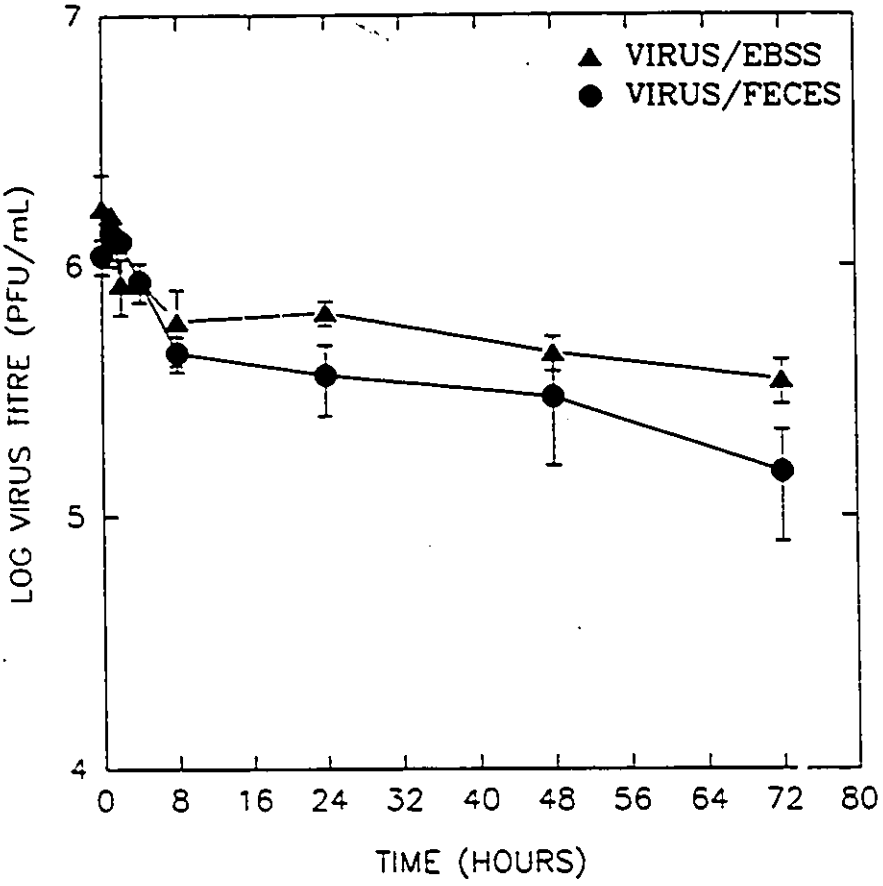

VIRUS/EBSS
VIRUS/FECES
LOG VIRUS TITRE (PFU/mL)
7
6
5
4
0
8
16
24
32
40
48
56
64
72
80
TIME (HOURS)

Figure 12. Survival of rHAd-RG1 on stainless steel disks under high RH (75±5%) and room temperature (23±2°C). The virus was suspended in either EBSS or 10% fox feces.

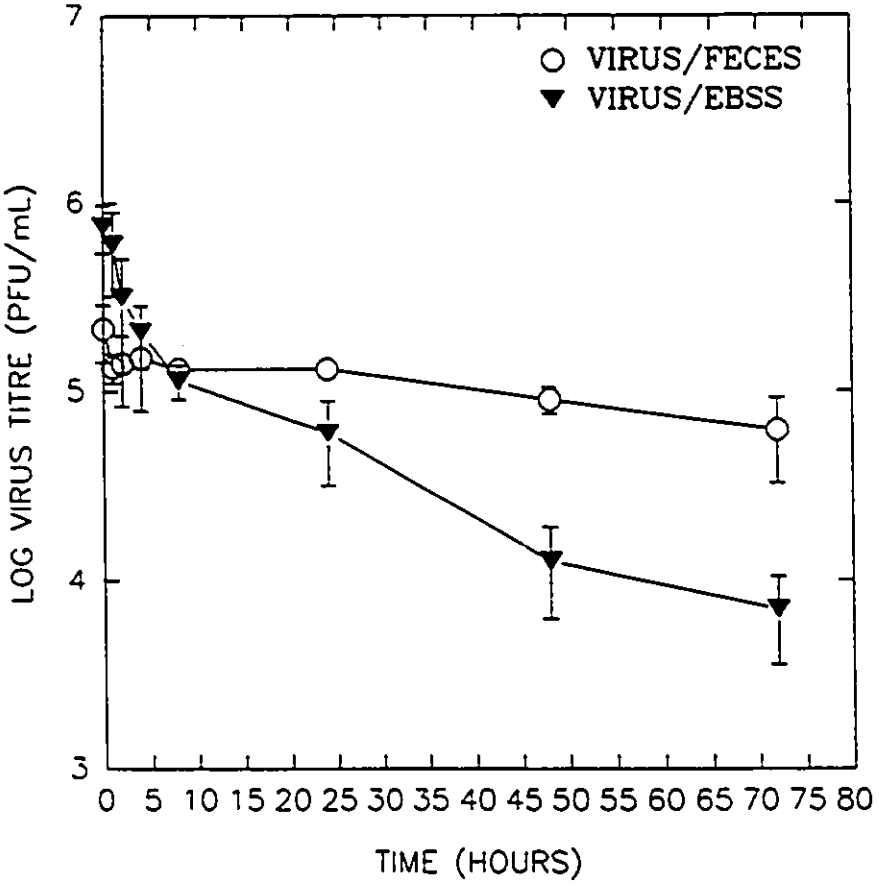

VIRUS/FECES
VIRUS/EBSS
LOG VIRUS TITRE (PFU/mL)
7
6
5
4
3
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
TIME (HOURS)

Table 5. Estimated half-lives and K_i values of rHAd-RG1 on stainless steel disks under different levels of relative humidity and when suspended in either EBSS or feces.

SUSPENDING MEDIUM (RH level)	K_i	CORRELATION COEFFICIENT	HALF-LIVES (DAYS)
Feces:			
low (20±5%)	0.0119	0.83	2.4
ambient (45±5%)	0.0184	0.86	1.6
high (80±5%)	0.0054	0.92	5.3
EBSS:			
low (20±5%)	0.0076	0.92	3.8
ambient (45±5%)	0.0057	0.70	5.0
high (80±5%)	0.0267	0.95	1.1

to decontaminate nonporous surfaces and instruments.

Chemicals such as ethanol and glutaraldehye are often used as fixatives. Was it, therefore, likely that the inability to detect infectious virus after disinfectant treatment was due to the fixation of viable virus to the disks rather than its inactivation? A series of experiments was conducted to answer this question.

The recombinant virus was radiolabelled with ^{35}S-methionine. The labelled virus was suspended in 5% fetal bovine serum and placed on the metal disks. The inoculum was allowed to dry and the disks were exposed to either EBSS (control) or the disinfectant under test as described above. The eluates were subjected to scintillation counting as well as plaque assayed.

The amount of radioactivity recovered after the treatment of the dried inoculum to 20 µL of EBSS was taken as the base-line and the recoveries after disinfection treatment were compared to it. A summary of these data from three separate experiments are presented as the percentage recovery of the radiolabel in Fig. 13. Elution of the inoculum after treatment with 70% ethanol and 2% glutaraldehyde gave recoveries of 90%; the recovery after Lysol Disinfectant Spray was 93%. These data clearly show that the elution procedure used in this study was highly efficient in the recovery of the dried and disinfectant treated inocula from the metal disks.

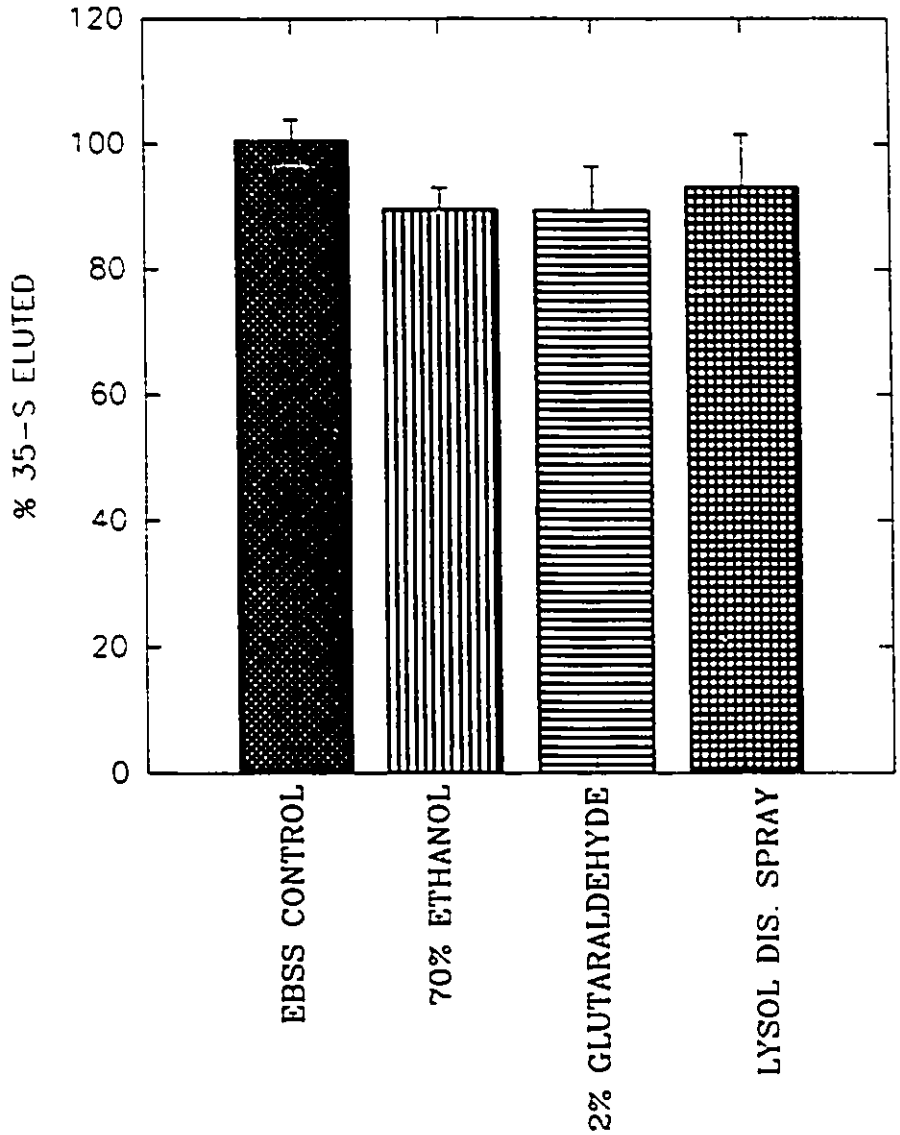
Table 6. Comparative efficacy of selected disinfectants against rHAd5-RG1 dried on stainless steel disks.

TREATMENTS	LOG VIRUS TITRE[1] (PFU/mL)
virus control, 0 min	7.8×10^5
virus control, 1 hr	3.4×10^5
Lysol Disinfectant Spray	No PFU detected
70% ethanol	" " "
2% glutaraldehyde	" " "
1% domestic bleach	" " "
EBSS (disinfectant ctrl)	1.5×10^5

[1] Arithmetic mean of viral titre

Ten microliters of rHAd5-RG1 were added to each stainless steel disk and allowed to dry; 20 µL of the respective disinfectants was added to each of the virus contaminated disks for 10 min at room temperature (23±2°C); disks were added to 980 µL EBSS, sonicated and titrated. Each sample was duplicated and the experiments were repeated twice.

Figure 13. The elution of ^{35}S-labelled virus from stainless steel disks after drying, disinfectant treatment or EBSS treatment.


120
100
80
60
40
20
0
% 35-S ELUTED
EBSS CONTROL
70% ETHANOL
2% GLUTARALDEHYDE
LYSOL DIS. SPRAY

5. SURVIVAL OF rHAd5-RG1 UNDER OUTDOOR CONDITIONS

These experiments were designed to determine virus survival when it is exposed to field conditions prior to its ingestion by target animals. The virus, suspended in EBSS, was added to blister packs which were embedded into the bait moulds (Fig. 1 & 2). The baits were then kept in a sun-exposed area or a shaded area for a period of 32 days. Samples were collected throughout the course of the experiment and titrated at the end of the experiment. Two sets of experiments were conducted, both in the latter part of the fall of 1990 and the fall of 1991.

The air temperature during these times ranged from 0-20^{0}C with the bait temperature usually 3^{0}C higher than the air temperature. The relative humidity ranged from 40-100% (Fig. 15 & 17). Data from the first field exposure (fall 1990; Fig. 14) showed that the virus was more stable in the shade than in the sun. Similar results were obtained when the second field exposure was conducted (Fall 1991; Fig. 16). The overall drop in virus titre in both the field trials was less than 1 $\log_{10}$ (Fig. 14 & 16). This suggests that the virus is stable enough to survive field exposure for 32 days.

The recombinant virus was suspended in several types of readily available substances to determine if virus stability could be enhanced in the field. Baits were prepared with the virus suspended separately in peptone, yeast extract, lactalbumin hydrolysate, tryptose phosphate broth and EBSS (control) and exposed to outdoor conditions under the sun during the first field trial (Fall 1990).

Figure 14. Survival of rHAd5-RG1 under outdoor conditions in the Fall of 1990.

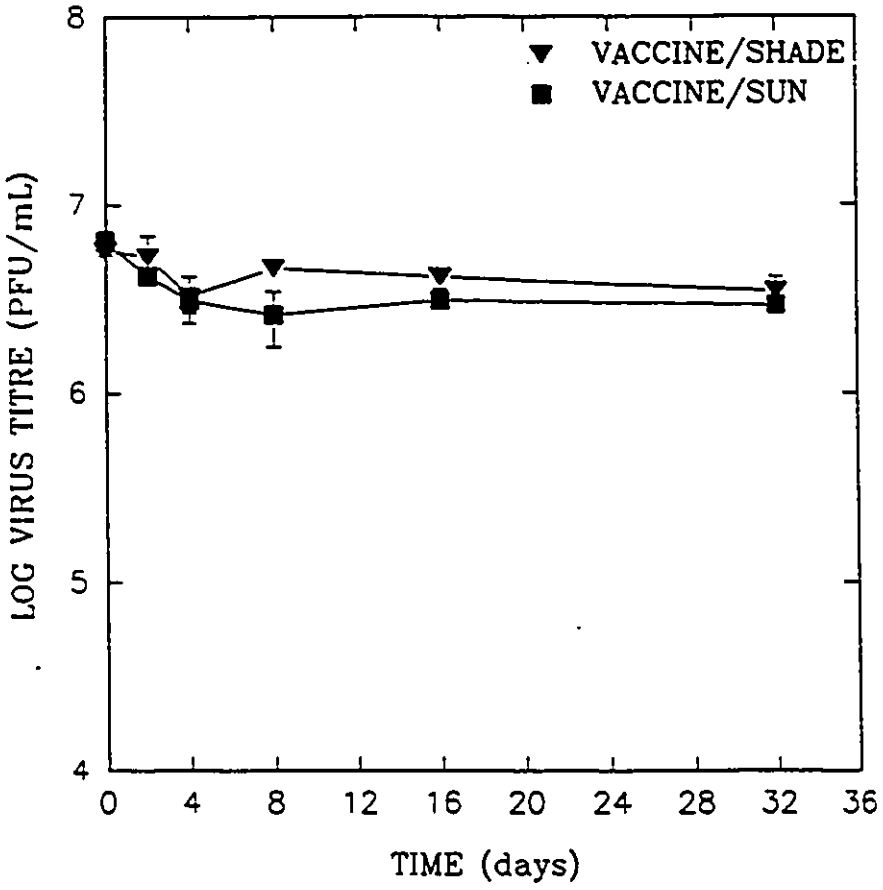

VACCINE/SHADE
VACCINE/SUN
LOG VIRUS TITRE (PFU/mL)
TIME (days)
8
7
6
5
4
0
4
8
12
16
20
24
28
32
36

Figure 15. Fluctuations in RH, air and bait temperatures during the outdoor survival experiment of rHAd5-RG1 in the Fall of 1990.

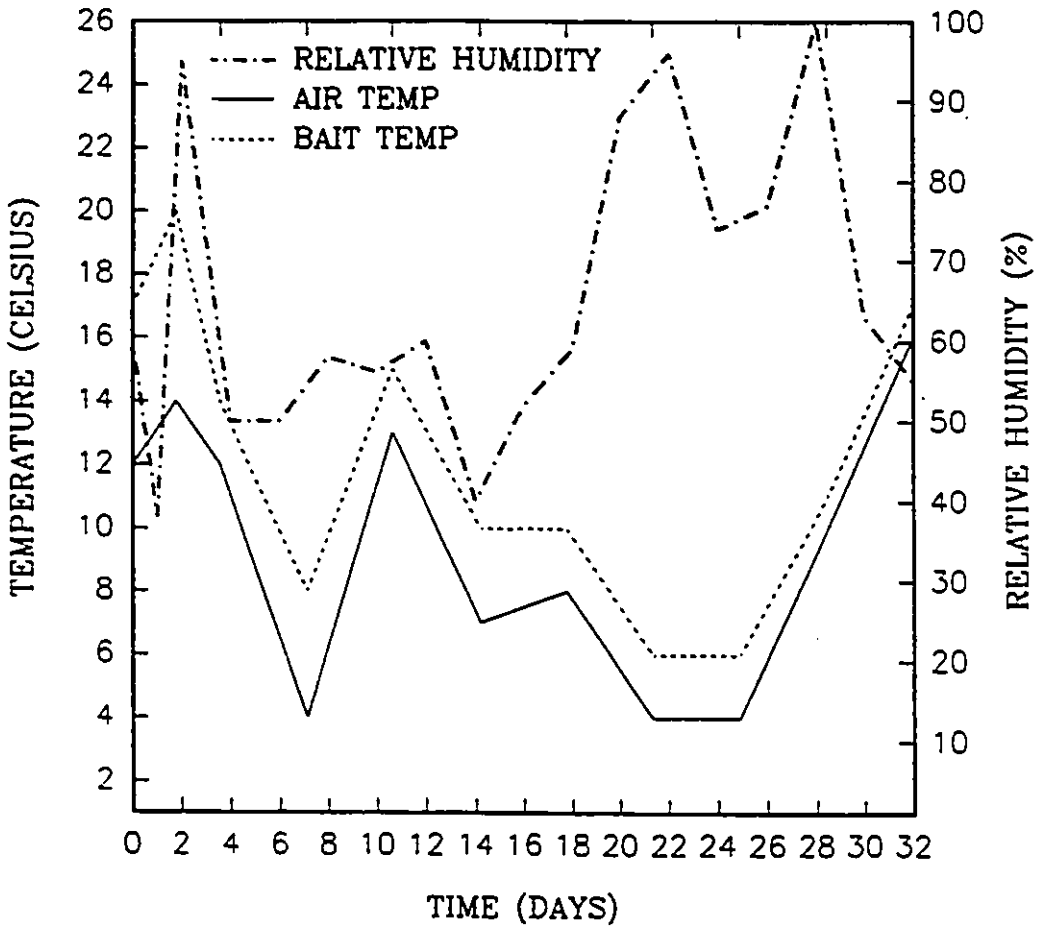

RELATIVE HUMIDITY
AIR TEMP
BAIT TEMP
TEMPERATURE (CELSIUS)
RELATIVE HUMIDITY (%)
TIME (DAYS)
26
24
22
20
18
16
14
12
10
8
6
4
2
100
90
80
70
60
50
40
30
20
10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32

Figure 16. Survival of rHAd5-RG1 under outdoor conditions in the Fall of 1991.

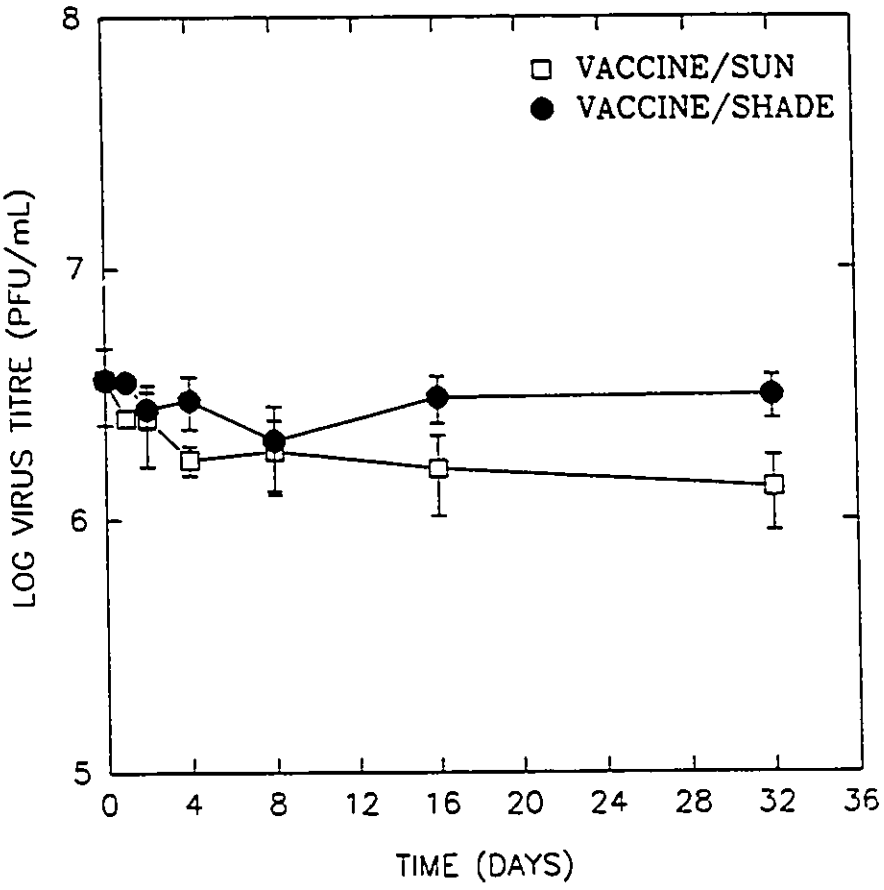
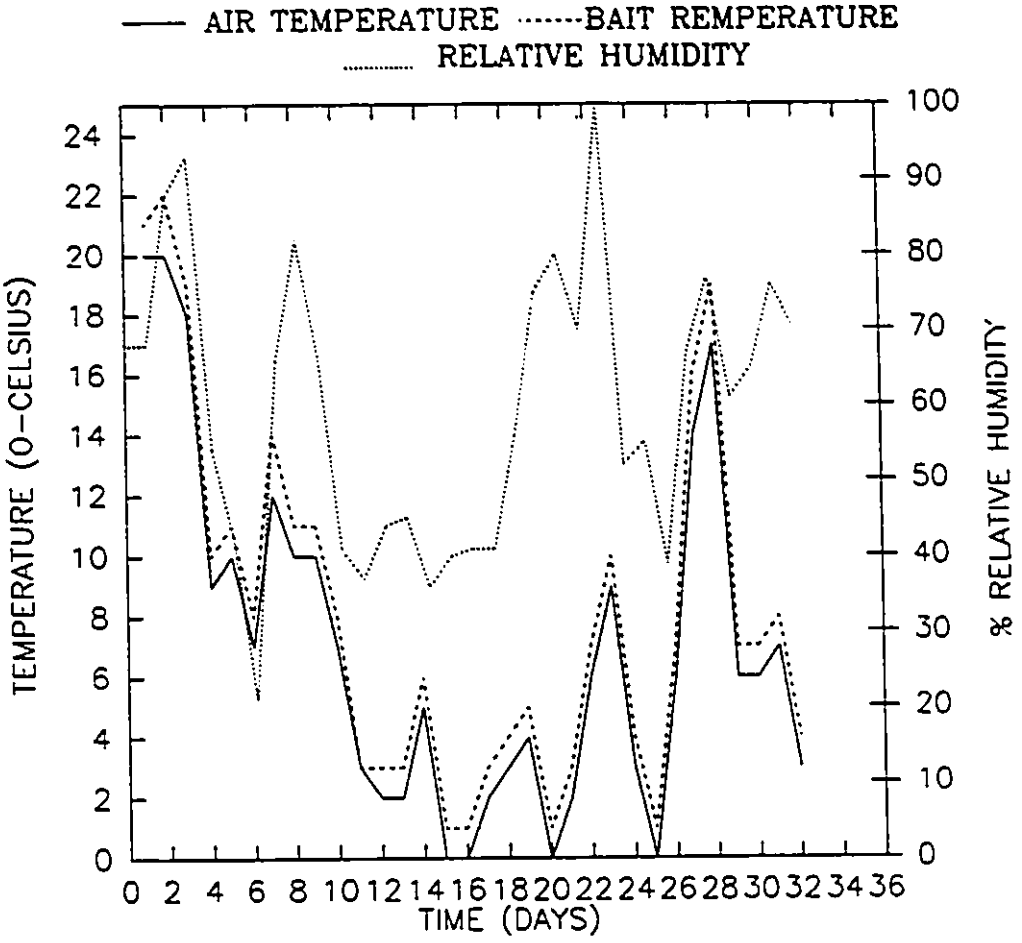

VACCINE/SUN
VACCINE/SHADE
LOG VIRUS TITRE (PFU/mL)
8
7
6
5
0
4
8
12
16
20
24
28
32
36
TIME (DAYS)

Figure 17. Fluctuations in RH, air and bait temperatures during the outdoor survival experiment of rHAd5-RG1 in the Fall of 1991.


AIR TEMPERATURE
BAIT REMPERATURE
RELATIVE HUMIDITY
TEMPERATURE (0-CELSIUS)
% RELATIVE HUMIDITY
TIME (DAYS)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
0 2 4 6 8 10 12 14 16 18 20 22 24
0 10 20 30 40 50 60 70 80 90 100

Samples were collected at the end of the 32-day period and plaque assayed along with the controls.

The results revealed differing degrees of stabilization of the virus by these substances (Fig. 18). A t-test ($\alpha = 0.05$) was used to determine if there was a significant decrease in viral titre before and after the field exposure. Peptone ($P = 0.56$) and yeast extract ($P = 0.18$) showed a stabilizing effect whereas the drop in virus titre was more pronounced in tryptose phosphate broth ($P = 0.003$), lactalbumin hydrolysate ($P = 0.006$) and vaccine control (EBSS)($P = 0.008$).

6. VIRUS RELEASE FROM INFECTED CELLS

Routine application of the recombinant virus in the field is dependent on the production of a high titered vaccine by efficient and economically viable procedures. Therefore, several technically simple methods to increase virus titre were first considered.

A large proportion of adenovirus infectivity remains cell associated (Hayashi and Russell, 1968) and it is lost during centrifugation procedures to clarify the cell harvest. In this study, mild detergents were used to release infectious virus from cellular materials as the first step to increase the virus titre in the vaccine.

In the initial experiments, Vero cells were used to grow the virus in 75 cm^2 flasks. Later on, the virus was cultivated in MRC-5 cells grown in roller bottles to confirm and extend the data obtained in the initial trials. Virus-infected cells were treated with the chemical under test and plaque assays were conducted

Figure 18. The effects of peptone, tryptose phosphate broth (Try. P. B.), yeast extract (Yeast ext.), and lactalbumin hydrolysate (Lact/Hy) in stabilizing the infectivity of rHAd5-RG1 under outdoor conditions.

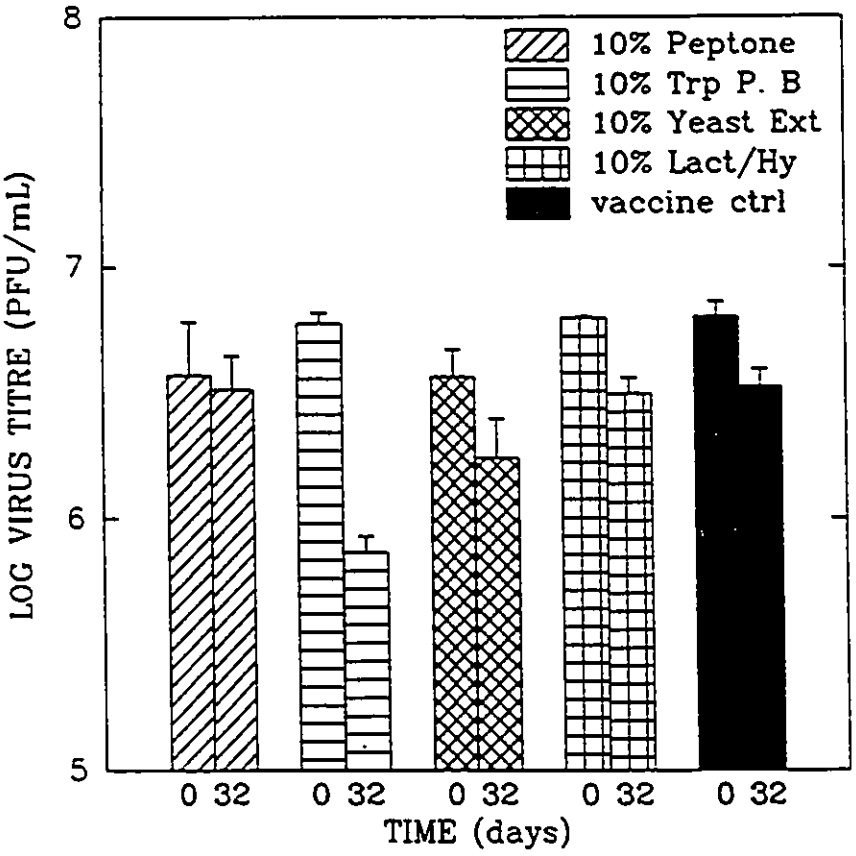

8
7
6
5
LOG VIRUS TITRE (PFU/mL)
10% Peptone
10% Trp P. B
10% Yeast Ext
10% Lact/Hy
vaccine ctrl
0 32
0 32
0 32
0 32
0 32
TIME (days)

Table 7. Relative increase in rHAd5-RG1 titre using either detergents, lysis buffers or a solvent.

TREATMENT	CHANGE IN INFECTIOUS VIRUS YIELD (±SD)
untreated cell suspension	1.0
0.1% NP-40	1.3X (0.2)
0.1% Triton-X100	1.6X (0.3)
0.1% CHAPS	3.5X (0.3)
0.1% sodium deoxycholate	5.5X (0.2)
equal-part Freon	4.5X (0.2)
lysis buffer A*	3.4X (0.1)
lysis buffer b^	5.9X (0.1)

* 1.5% NP-40, 20 mM $MgCl_2$

^ 1% NP-40, 1% Triton-X100, 50 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5

rHAd5-RG1 infected MRC-5 cells were treated with the above mentioned solutions in their final concentration for 30 min at 4°C; samples were titrated before and after treatment.

on treated and untreated (control) preparations and the increase in virus titre calculated.

As can be seen in Table 7, Lysis buffer B resulted in the highest increase with a 5.9-fold difference over the untreated samples. For the detergents, 0.1% sodium deoxycholate gave a 5.5-fold increase in the infectivity titre over untreated samples. The highest virus titre achieved using the detergents were 2.5 X 10^8 PFU/mL.

7. VIRUS CONCENTRATION BY HYDROEXTRACTION

To concentrate the virus further, hydroextraction using polyethylene glycol (PEG) and polyacrylamide was attempted. The detergent-treated preparations were hydroextracted and the efficiency of virus recovery was calculated after plaque assays.

The results (Table 8) show that PEG could concentrate the virus 10-fold with a recovery efficiency of 51-66%. In contrast to this, the polyacrylamide gave a calculated efficiency of recovery of 26-38% with a 10-fold reduction in the sample volume. Thus PEG proved to be superior for virus concentration.

8. MICROENCAPSULATION OF BSA AND OF rHAd5-RG1

One disadvantage with oral attenuated viral vaccines is that they are often susceptible to the pH extremes as well as the enzymatic and detergent activity encountered in the digestive tract. Microencapsulation of the virus is one way to protect it from inactivation before it reaches the target sites. It should be noted here that the exact point(s) for the antigenic stimulation of target animals by the

Table 8. Concentration of rHAd5-RG1 by hydroextraction using either polyethylene glycol 8000 or polyacrylamide.

	BEFORE CONCENTRATION			AFTER CONCENTRATION				
	VOLUME	VIRUS TITRE		VOLUME CALCULATED		VIRUS TITRE		
METHOD	mL	PFU/mL	Total PFU	mL	conc. factor	PFU/mL	Total PFU	calc. efficiency
PEG	50	$7.7X10^6$	$3.8X10^8$	5	10	$4.4X10^7$	$2.2X10^8$	58%
	50	$1.6X10^6$	$8.7X10^7$	5	10	$9.0X10^6$	$4.5X10^7$	51%
	50	$1.0X10^6$	$5.0X10^7$	5	10	$6.6X10^6$	$3.3X10^7$	66%
Polyacrylamide	50	$6.8X10^6$	$3.4X10^8$	5	10	$2.7X10^7$	$1.3X10^8$	38%
	50	$3.5X10^6$	$1.8X10^8$	5	10	$9.3X10^6$	$4.7X10^7$	26%
	50	$1.6X10^7$	$8.8X10^8$	5	10	$6.0X10^7$	$3.0X10^8$	34%

recombinant adenovirus are not yet identified. Availability of microencapsulated virus may help to design and conduct experiments to elucidate this issue. In view of this, attempts were made to coat the virus with either cellulose acetate phthalate (CAP) or sodium alginate.

As can be seen from Fig. 3, the CAP microcapsules were very rough and chalk-like in appearance. They were uneven with their size ranging from 3 to 5 mm in diameter. On the other hand, the sodium alginate microcapsules (Fig. 4) were translucent, spherical and very smooth in appearance. They were also quite uniform in size (approximately 3 mm in diameter).

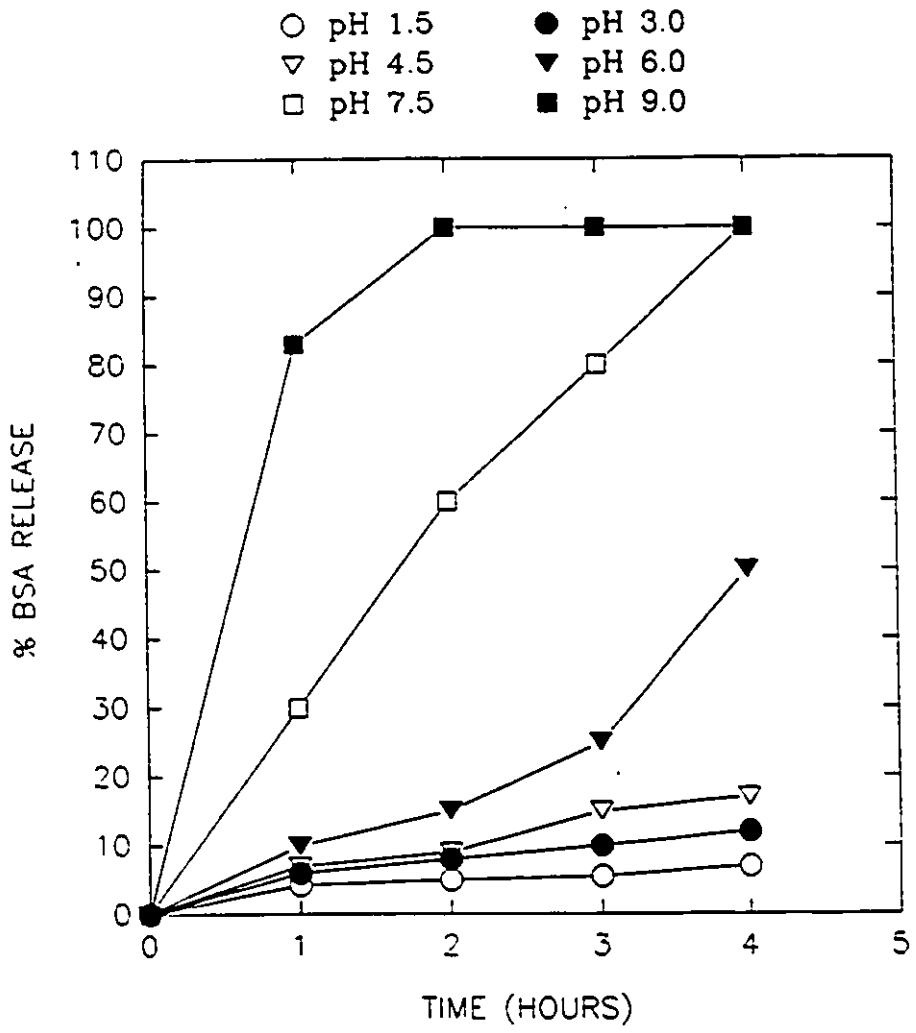
In the following experiments, the controls consisted of unencapsulated material prepared as (a) for CAP, labelled rHAd5-RG1 lyophilized with sucrose and corn starch, and (b) for sodium alginate, a mixture of labelled but unlyophilized rHAd5-RG1, sucrose and corn starch.

8.1 Cellulose acetate phthalate microencapsulation

To determine the pH resistance of CAP-encapsulated material, the initial experiments were conducted with bovine serum albumin (BSA) as the core material. The microcapsules were exposed to pH ranging from 1.5-9.0 and the release of BSA was tested spectrophotometrically.

As shown in Fig. 19, the core material was released in a pH dependent manner, with less than 20% detected at pH values of 1.5, 3.0, 4.5 after 4 hr; at pH 6.0, nearly 50% of the BSA became released. However, at pH 7.5 nearly all of the microencapsulated BSA became detectable in solution within 4 hr. The release of

Figure 19. Release of bovine serum albumin (BSA) encapsulated in cellulose acetate phthalate.


○ pH 1.5
● pH 3.0
▽ pH 4.5
▼ pH 6.0
□ pH 7.5
■ pH 9.0
% BSA RELEASE
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
TIME (HOURS)

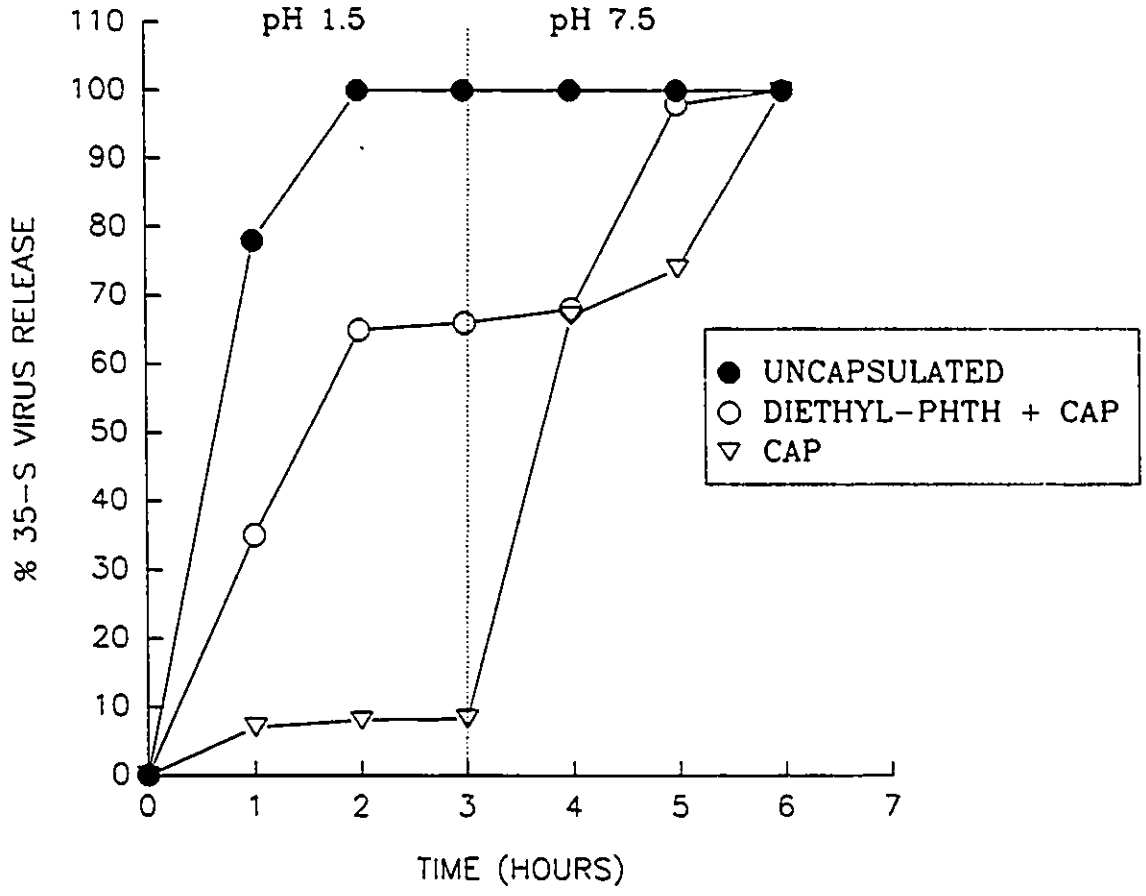
all of the BSA occurred in only 2 hr at pH 9.0. These results clearly indicate the breakdown of CAP at alkaline pH which makes it suitable for enteric coating. Additional experiments were therefore conducted with ^{35}S-labelled rHAd5-RG1 encapsulated in CAP.

The release of the labelled virus was monitored in simulated gastric (pH 1.5) and intestinal (pH 7.5) juices prepared according to USP. As can be seen from Fig. 20, only 10% of the labelled and encapsulated virus was released in 3 hr at pH 1.5. But, when the microcapsules were transferred to the intestinal juices, almost all the radioactivity became detectable in solution within the next 3 hr. All of the unencapsulated material was released within the first 2 hr at pH 1.5.

Diethyl phthalate is often used as a plasticizer in the encapsulation of pharmaceuticals (Metha 1986). We therefore, incorporated it in the coat material with CAP and tested the effects of pH on the release of the core material. The presence of the plasticizer considerably reduced the resistance of the microcapsules to the acidic pH (Fig. 20), since more than 60% of the core material became detectable in solution within the first 2 hr.

The release of the core material was also monitored by plaque assay for rHAd5-RG1. The amount of infectious virus in 0.1 gm of the lyophilized material used for encapsulation was 7.0×10^4 PFU. The total number of PFU detected in 20 mL of the simulated intestinal juice at the end of the experiment was 2.0×10^3 PFU. Therefore, only 3.0% of the input infectious virus was recovered.

Figure 20. Release of cellulose acetate phthalate encapsulated rHAd5-RG1 in simulated gastric juices (pH 1.5) and intestinal juices (pH 7.5)(dashed line indicates adjustment of pH).

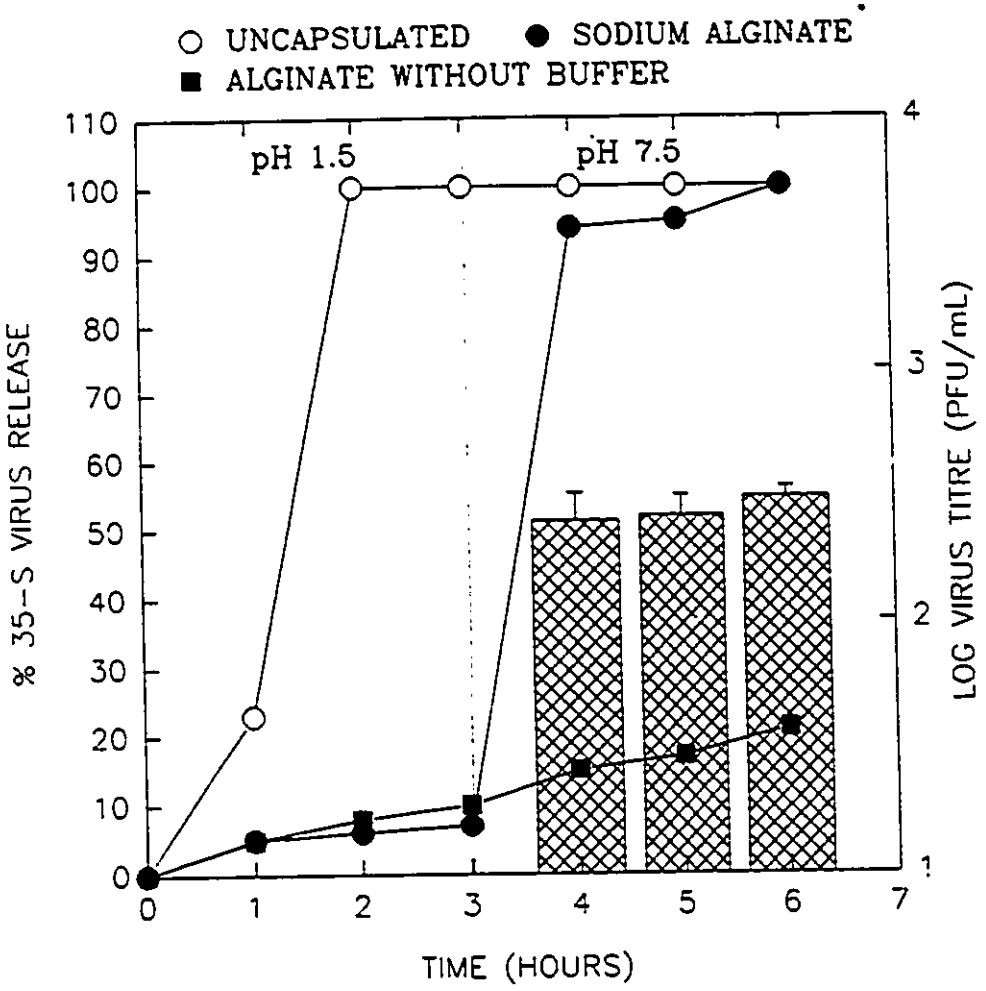

pH 1.5
pH 7.5
110
100
90
80
70
60
50
40
30
20
10
0
% 35-S VIRUS RELEASE
0
1
2
3
4
5
6
7
TIME (HOURS)
UNCAPSULATED
DIETHYL-PHTH + CAP
CAP

8.2 Sodium alginate microencapsulation

In the initial experiments with the sodium alginate encapsulated virus, the release of the core material appeared to be pH dependent (Fig. 21). However, it was suspected that the potassium ions present in the intestinal juices but absent in the gastric juices (Maharaj *et al.*, 1984) may have accounted for the observed pattern of release. Additional experiments were, therefore, carried out to confirm the results using double distilled water at pH 1.5 and 7.5 as the suspending medium for the microcapsules. Only 20% of the encapsulated material was released even after 6 hr of exposure to the two pH levels suggesting a role for potassium ions in the virus released observed earlier.

Release and recovery of the encapsulated virus was better from the sodium alginate microcapsules as compared to that from CAP (Fig. 21). The initial virus titre in the mixture before encapsulation was 6.0×10^4 PFU/mL. No infectious virus was detected in the supernatant at the end of 3 hr while the microcapsules were still at low pH. However, nearly 22% of the input PFU could be recovered in the supernatant 1 hr after the microcapsules were transferred to the simulated intestinal juices; the level of detectable PFU became 25% after an additional 2 hr.

Figure 21. Release of sodium alginate encapsulated rHAd5-RG1 in simulated gastric juices (pH 1.5) and simulated intestinal juices (pH 7.5)(dashed line indicates the adjustment of pH).


○ UNCAPSULATED
● SODIUM ALGINATE
■ ALGINATE WITHOUT BUFFER
pH 1.5
pH 7.5
% 35-S VIRUS RELEASE
LOG VIRUS TITRE (PFU/mL)
TIME (HOURS)
0
10
20
30
40
50
60
70
80
90
100
110
1
2
3
4
5
6
7

DISCUSSION

Wildlife rabies continues to be a problem in Ontario with foxes and skunks being the major reservoirs at the present time (Black and Lawson, 1973; Lawson *et al.*, 1988; Tolson *et al.*, 1988). Raccoon rabies has now reached northern parts of New York State and it is quite likely that this outbreak will extend into Ontario in the near future (Winkler and Jenkins, 1991). Development of methods to control the spread of rabies in wildlife is therefore among the present priorities of the Ontario Ministry of Natural Resources (MacInnes *et al.*, 1988). The development of vaccines for the immunization of wildlife against rabies is one of these approaches. The success of rabies control in certain parts of Europe through the oral vaccination of wildlife (Steck *et al.*, 1982; Schneider *et al.*, 1988) attests to the validity of such an approach.

As stated earlier, the attenuated rabies virus (ERA), currently being used in the oral vaccination campaigns of wildlife in Ontario has several drawbacks. The recombinant adenovirus (Prevec *et al.*, 1990) tested in this study is being considered as a possible replacement for the attenuated rabies virus. Investigations at the Animal Disease Research Institute showed that the recombinant virus can immunize foxes and skunks against rabies when given by the oral route (Charlton *et al.*, 1992). The suitability of the virus as an oral vaccine for raccoons remains to be determined. Information on the stability of the virus under indoor and outdoor conditions was required to determine its suitability for vaccine manufacture, storage and field trials. Such an investigation was deemed particularly crucial since rHAd5-RG1 is a genetically engineered virus and

information on its survival would be necessary to satisfy the regulatory requirements.

1. SURVIVAL OF rHAd5-RG1 UNDER INDOOR CONDITIONS

Many live vaccines contain a chemical stabilizer that is meant to protect vaccine potency during its shipment and storage (Burfoot *et al.*, 1977; Frerich and Herbert 1974). The Connaught Laboratories stabilizer was found to reduce the titre of the virus (Fig. 5). The inhibitory activity appeared to be due to the gelatin and egg yolk contained in it, whereas the buffer (Tris-HCl) in itself did not interfere with the plaque assay. The inhibitory components in the stabilizer may have interfered directly by combining with the virus or indirectly by blocking its attachment to the host cells; aggregation of virus particles may be another reason for the lower infectivity titre. On the other hand, the increased virus quantitated in the presence of Tris-HCl could have been as a result of the break-up of the viral clumps (Kovacs, 1964).

Since the virus appeared to be fairly stable in EBSS alone at both room temperature and under refrigeration, the addition of a stabilizer is not considered necessary. This should make vaccine manufacture cheaper and simpler because egg yolk for stabilizers must be obtained from pathogen-free flocks. This limits its availability and adds to quality control measures.

The results of the thermostability tests on the virus suspended in EBSS showed it to be reasonably stable even at 37°C (Table 4 and Fig. 8). This suggests that the exposure of the vaccine to summer temperatures in temperate regions

may not reduce its potency for several days. The vaccine may also be suitable in tropical settings where access to refrigeration facilities for its storage is generally limited.

Tests of the comparative thermostability of the recombinant virus, its parent and a wild-type human adenovirus type 5 clearly demonstrate that neither the insertion of the rabies glycoprotein gene nor the E3 deletion has altered the capacity of these viruses to retain their infectivity at 37°C. This temperature was selected to simulate accelerated degradation of the vaccine (Jerne and Perry, 1956). Even though the comparative survival of the three viruses was not tested at room or refrigeration temperatures, it is unlikely that their behaviour would prove to be different.

The results of the comparative thermostability tests are significant from a regulatory point of view as well. Since the recombinant virus was not found to have acquired enhanced thermoresistance, such a genetically modified organism is unlikely to persist in the environment any longer than naturally occurring adenoviruses.

To minimize the loss of infectivity during the storage and shipment of attenuated vaccines, many manufacturers have resorted to the use of lyophilized products (Frerich and Herbert, 1974; Hekker *et al.*, 1973; Kurokawa *et al.*, 1979). The results of the recombinant virus lyophilization and storage have shown that the virus can readily withstand freeze drying and can be stored for at least a few weeks even at room temperature without any significant loss in its infectivity.

This should simplify considerably the storage and shipment of the vaccine because no refrigeration facilities would be required.

2. SURVIVAL OF rHAd5-RG1 ON ENVIRONMENTAL SURFACES

The ability of viruses to survive on environmental surfaces is influenced by RH levels (Mbithi *et al.*, 1991; Lloyd-Evans, 1986; Parker *et al.*, 1944). Previous studies on the influence of RH with respect to adenovirus survival on contaminated surfaces have generated contradictory results. Selwyn (1965) and Mahl and Sadler (1975) showed that higher levels of RH favoured the survival of adenovirus types 3 and 2, respectively. More recent findings of Nauheim *et al.* (1990) and Hara *et al.* (1990) showed the reverse to be true; adenovirus type 19 was found to survive better at the lower levels of RH. These differences may be due to the type and strain of virus used and the nature of the virus suspending medium.

Initially, attempts were made to obtain fecal samples from foxes and skunks which were orally exposed to the recombinant virus at the Animal Disease Research Institute. Nearly twelve such samples were tested and none of them was found to contain sufficient infectious virus using Vero cells. In view of this, samples of feces from unvaccinated foxes and skunks were experimentally contaminated with the recombinant virus and used for the inoculation of the metal disks. A 10% fecal suspension was used because higher concentrations of the feces were cytotoxic to the cells and gave erratic results. Stainless steel disks were selected as representative nonporous environmental surfaces based on

earlier observations that virus survival on such metal disks was representative of that on plastic and glass (Sattar *et al.*, 1986).

The low (20±5%) and high (80±5%) RH levels selected in this study are representative of the winter and summer seasons, respectively. The medium (45±5%) RH level is generally encountered in a controlled environment.

No clear cut pattern of the influence of RH on virus survival was observed in this study. The reasons for this are not apparent at this stage. Irrespective of the type of suspending medium and the level of RH, the virus remained detectable on the metal disks for at least 3 days. This should be borne in mind when handling surfaces and objects contaminated with the virus.

3. CHEMICAL DISINFECTION OF rHAd5-RG1

Nonenveloped viruses are generally more resistant to commonly used hard-surface disinfectants than enveloped viruses (Lloyd-Evans *et al.*, 1986; Hoff and Akin, 1986; Mbithi *et al.*, 1990; Springthorpe and Sattar 1990). In this study, 5% fetal calf serum was used as the organic load in accordance with the guidelines for virucidal tests of the US Environmental Protection Agency (1981) and the standards for germicidal tests published by the Canadian General Standards Board (1990).

All of the tested disinfectants were able to completely inactivate the recombinant virus within 10 min at room temperature. Earlier studies showed a wild-type human adenovirus type 5 to be susceptible to these disinfectants (Sattar *et al.*, 1989). This suggests that the genetic manipulations of the virus have not

increased its resistance to the types of disinfectants tested, thus indicating that any of the tested products can be used in decontaminating hard surfaces where the recombinant virus is being handled.

Although a number of the tested disinfectants in this study are commonly used as fixatives (Springthorpe and Sattar, 1990), the results summarized in Fig. 13 clearly show that the elution procedure used was highly efficient in recovering the labelled material from the disks even after exposure to ethanol, glutaraldehyde and the Lysol Disinfectant Spray. Sodium hypochlorite (domestic bleach) is not a fixative but a strong oxidizing agent and the elution procedure was able to recover nearly all of the dried and labelled inoculum even after treatment with the bleach (data not shown).

4. SURVIVAL OF rHAd5-RG1 IN THE FIELD

The recombinant virus is a candidate primarily for the oral vaccination of wildlife and the ultimate test of its stability will be in the field. However, the two outdoor exposures conducted with the bait-coated recombinant virus in this study closely simulated the conditions it may encounter after being dropped from low flying airplanes. Since there was no appreciable drop in the infectivity titre of the virus even when exposed to the sun, the recombinant shows strong potential for field application. In both the field trials the air temperature did not go above 20°C, but the virus survival data from indoor experiments suggests that it should be able to withstand higher temperatures.

The protein stabilizers tested gave varying results and even the most

pronounced stabilizing effect was not appreciably greater than that of EBSS alone. Whereas all the substances tested are relatively inexpensive, safe and readily available, addition of such extra components to the vaccine is not considered necessary.

4. GROWTH AND CONCENTRATION OF rHAd5-RG1

Adenovirus virions normally mature in the nucleus (Horwitz *et al*, 1969) where they remain cell associated well after virus synthesis (Green and Pina, 1963). Furthermore, human adenovirus type 5 can infect many different cell lines but the outcome can range from an abortive to a productive infection (Nevins, 1987). MRC-5 cells were used for growing the virus tested in this study because the seed lots of the recombinant have also been prepared in this cell line. Even though these are not the most suitable hosts for the virus, they are licensed for vaccine production (Jacobs, 1976). In our hands, Vero cells proved to be the most suitable for virus titration either by plaque assay or $TCID_{50}$. The plaque assay was chosen for routine titration because of a greater degree of precision provided by it. The 293 cell line is considered very appropriate for adenovirus isolation and quantitation (Graham *et al*, 1977). We were unsuccessful in carrying this cell line on a regular basis and were also unable to get the recombinant to plaque in it. The use of other epithelial cell lines of human origin (HEp-2, HeLa) for plaque assay also proved to be unsatisfactory. Recently, some of the problems with cell cultures in this laboratory have been found to be related to the use of chemicals in the steam for our autoclaves.

In this study, the methods used to grow and concentrate the virus were selected for their relative simplicity, economy and potential for application in the commercial production of the vaccine.

For virus production, the cells were grown in corrugated roller bottles which provided a large surface area per unit volume of the medium. However, the MRC-5 cells were found to grow rather slowly in these bottles with complete monolayer formation taking as much as 7 days and this appeared to reduce the susceptibility of the cells to the virus. Therefore, the monolayers were infected at an estimated 75% confluency. Since it was difficult to determine the exact number of cells in such a roller bottle, the ratio of the virus PFU in the inocula to the number of cells was estimated to be about 0.1. The corrugated surface of the bottles did not permit the complete recovery of the infected cells. Therefore, further work will be required to adapt their use in the large scale production of the virus. Preliminary experiments using microcarrier beads (Cytodex 1; Pharmacia) for culturing MRC-5 cells showed this system to be potentially suited for the production of the recombinant for vaccine manufacture.

Centrifugation is a problematic procedure for application in commercial the production of any vaccine. It is considered particularly unsuitable in dealing with cell culture harvests of adenoviruses because a large proportion of the virus is cell-associated and would be lost during removal of cell debris. In view of this, the use of mild detergents to dissolve cell membranes was attempted and the testing of certain detergents alone or in combination has given encouraging

results. However, the following factors must be considered before their regular application in vaccine manufacture:

Recent studies by Gaudin *et al.* (1992) show that the rabies glycoprotein exists as a trimer and only CHAPS did not alter this structure. This is important, since the induction of protective immunity depends on the glycoprotein maintaining its native structure. The use of detergents may also interfere with the immunogenic potential of the oral vaccine. Such a vaccine preparation may be unpalatable or toxic to the target species. Further *in vivo* testing is therefore important before the use of detergents can be incorporated in vaccine manufacture, especially for field use.

The use of PEG hydroextraction is a relatively inexpensive and technically simple way of concentrating the recombinant virus (Ramia and Sattar, 1979) and is potentially suited for scaling up. The reasons for the lower yields of infectious virus in the polyacrylamide procedure are not clear but may be due to the adsorption of the virus to the beads.

5. MICROENCAPSULATION OF BSA AND rHAd5-RG1

Microencapsulation is widely used for coating various types of pharmaceuticals (Metha, 1986). CAP, which is a physiologically inert polymer, is commonly used as an enteric-coating (Lin and Kawashima, 1987; Madan and Shanbhag, 1978; Merkle and Speiser, 1973). The process of microencapsulation with CAP requires either heat treatment of organic solvents such as acetone-ethanol and chloroform. The use heat in working with infectious agents is

undesirable and in an earlier study (Maharaj *et al.*, 1984) the organic solvents inactivated the attenuated rabies virus during CAP encapsulation. However, the recombinant virus was found to withstand exposure to the solvents and this allowed us to proceed with the encapsulation process.

Proper encapsulation using CAP requires high-speed stirring of the mixture. Since we did not have access to such a device, the quality of microencapsulation was not entirely satisfactory and this was reflected not only in the wide variation in the size and shape of the capsules but also in the low levels of incorporation of infectious virus in them. The addition of diethyl phthalate also did not improve CAP encapsulation in our hands. Sodium alginate, the other material tested in this study, is used in the encapsulation of mammalian cells and enzymes (Sinamore *et al*, 1989).

The suitability of encapsulated virus for the oral immunization of wildlife remains to be tested. Should it prove successful, it may become possible to mix the microcapsules with the bait material directly thereby avoiding the need for the blister packs. It should be noted here that the actual site of antigenic stimulation in the orally immunized animals remains to be determined. Limited data from direct deposition of the virus in the intestine suggest that this is not the primary site for antigenic stimulation (Wandeler, personal communication.) The use of enteric coated virus may help to resolve this issue. If animals challenged with such encapsulated material do not show seroconversion, it will strengthen the view that the oral cavity is where the primary antigenic response occurs.

CONCLUDING REMARKS AND DIRECTIONS FOR THE FUTURE

This two-year study has shown that rHAd5-RG1 is stable enough both indoors and outdoors to be used as an oral vaccine for wildlife. At the same time, the deletion of the E3 region and the insertion of the rabies glycoprotein gene did not appear to change the recombinant's stability in a way to make it unsuitable for field application. More recently, other adenovirus recombinants have been obtained which express the rabies glycoprotein gene more efficiently (Prevec, personal communication). Whether these are more suitable for the immunization of wildlife remains to be determined. Furthermore, studies similar to those reported here will be required to determine the relative stability of other recombinants.

rHAd5-RG1 is excreted in the feces of the orally immunized animals, but it is yet to be determined if the excreted virus is the progeny or the input virus itself. Antigenic stimulation of the exposed animals without extensive virus replication in their bodies is perhaps desirable because it will reduce the chances of environmental contamination and spread of the recombinant to nontarget species. This will also minimize the possibility of genetic transfer between the recombinant and other microorganisms.

It is considered desirable to have a titre of 10^9 PFU/mL of the virus in the oral vaccine. Our attempts to consistently obtain virus pools with such a titre were unsuccessful. The reasons for this are not clear but the problems with the quality of the steam for autoclaving may have been a factor.

As far as we are aware, little is known on the comparative stability of

genetically engineered viruses of human or animal origin. Therefore, the study described here is among the first of its kind and the protocol developed in this investigation should help in future work.

Work of this nature is increasingly important since many recombinants will be added to the environment.

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