AN ATTENUATED MINK ENTERITIS VIRUS AND ITS USE IN A TRIVALENT VACCINE: STUDIES ON SAFETY AND ANTIGENICITY

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INTRODUCTION
Mink enteritis is a highly contagious disease of mink caused by a parvovirus which is closely related, antigenically, to the virus which causes feline panleucopenia. The disease was first recognized by Schofield (7) in Fort William, Ontario, Canada, in 1947. Since then, it has been observed in many mink-ranching areas in Canada and the United States. The disease has also been reported in a number of other countries including Denmark, Finland, the Netherlands, Sweden and the United Kingdom. The development of a vaccine was first reported in 1952 by Wills (9). It consisted of a formalin treated suspension of liver and spleen from infected mink and was found to limit the morbidity and duration of the disease both under experimental conditions and in the field. Preparation of a similar, formalin-inactivated, mink enteritis vaccine was also described by Pridham and Wills (6) and Kangas (5).

Until 1968, the inactivated vaccine of mink-tissue origin was the only type of mink enteritis vaccine available. In 1967, Johnson (3) reported successful propagation of mink enteritis virus in primary feline kidney cell cultures. In 1968, the development of an attenuated mink enteritis vaccine using primary feline tissue culture cells as substrate was reported (10). Three years later Ackermann (1) reported the immunization of mink kits against mink enteritis using an attenuated feline panleucopenia virus and botulinum toxoid in a combined vaccine.

This paper reports the development of an attenuated, live-virus, mink enteritis vaccine, propagated in feline kidney tissue cell cultures, and on its subsequent combination with a mink distemper vaccine and Clostridium botulinum toxoid.

MATERIALS AND METHODS

Vaccines
The mink enteritis virus, originally isolated from tissues of infected mink, was serially passed eleven times in mink. A suspension of liver, spleen and intestines was prepared, centrifuged and the supernatant used as an inoculum for primary feline kidney cell cultures. The virus lost its pathogenicity for mink after serial passage in primary feline kidney cells.

The mink distemper vaccine used in the study contained an attenuated live virus. The original ferret-virulent virus was attenuated by serial passage in primary canine kidney cell cultures. This virus was further adapted to a canine kidney cell line.

Clostridium botulinum toxoid, type C, beta was a formalin-inactivated toxoid produced by Clostridium botulinum (CID South African Strain). The toxoid was subsequently adsorbed onto aluminium phosphate at a final concentration of 1%.

Challenge Procedures

The virulent mink enteritis challenge virus was stored at -20°C as a 40% suspension of virus-infected livers and spleens. The challenge material was diluted to a 10% suspension in phosphate buffered saline (PBS) for challenge purposes and administered by stomach tube in a 5 ml dose. Susceptible mink developed a complete anorexia accompanied by diarrhea, with the excretion of fibrinous gray, white or cream-coloured casts three to four days after challenge.

Virulent canine distemper virus (Snyder Hill Strain) which is antigenically identical to mink distemper was used as the mink distemper challenge. It was stored at -20°C as a 10% suspension of virus-infected dog brain. The challenge material was diluted to a 2% suspension in PBS and administered intraperitoneally in a 2 ml dose.

Susceptible mink on challenge exhibit photophobia, lacrimation, anorexia with the development of convulsions, usually during the third and fourth week after challenge. The sick mink may also develop dermatotropic signs, manifested by swelling, wrinkling, thickening and crustiness of the skin on the face.

A freeze-dried crude Clostridium botulinum toxoid was used for the botulinum challenge. The challenge material was diluted to 1:100 in a diluent containing 1% disodium phosphate.
and 0.2% gelatin in distilled water, pH 6.5, for challenge purposes and was administered orally by stomach tube in a 2 ml dose. Susceptible mink usually die 24 to 48 hours after challenge, and show varying degrees of paralysis and dyspnea.

**Titration of Mink Enteritis Virus**

Specific Haemagglutination Test (HA Test) – The haemagglutination of swine erythrocytes by the mink enteritis virus is a reliable test for titrating the virus (4). The haemagglutination can be inhibited by specific antibodies. Throughout this work the test was carried out in microplates. Erythrocytes collected in 2% sodium citrate solution were washed three times in veronal buffer and 0.05 ml of a 0.5-0.7% suspension was added to 0.05 ml amounts of twofold dilutions of virus.

A phosphate buffer (pH 6.8) was used as diluent. The optimum temperature for the test is 4°C and the time required for complete haemagglutination is six to twelve hours.

The titre obtained by haemagglutination can be compared with the standard, cytopathogenic effect (CPE) test (8) which is a widely used technique for the titration of mink enteritis virus. Our results, on a comparison of the HA and CPE tests, are comparable with those reported by Johnson (4), one HA unit being equivalent to 80 to 100 TCID<sub>50</sub>. Although, in our laboratory, the HA test is somewhat less sensitive than the CPE test, it has the advantages of ease of performance and better reproducibility.

**Tissue Culture Haemagglutination Test (TCHA)**

Secondary feline kidney cells were infected at the time of planting with tenfold dilutions of mink enteritis virus. Five to six days after infection, fluids harvested from these tissue cultures were tested for haemagglutination. This is a sensitive method for determining the actual concentration of live virus particles in the original material and is comparable with the CPE test.

**Isolation of Virus from Tissues and from Faecal Samples**

The mink tissues which consisted of spleen, liver, kidney and large intestine were ground up in a mortar giving 10% suspensions in PBS. The suspensions of tissue were left in the PBS for one to two hours to elute the virus from the tissue. The suspension was centrifuged for ten minutes at 350 g, the supernatant removed and penicillin and streptomycin added to concentrations of 200 units, and 200 micrograms per ml respectively. The supernatant was used to inoculate cultures of feline kidney cells for the isolation of virus.

Small pieces of mink feces were placed in liver vials containing five ml of 0.5% lactalbumin in Hanks’ balanced salt solution. After one to two hours at room temperature, the suspensions were centrifuged, the supernatants removed and the antibiotics added as above. The supernatants were used as the inoculum for the isolation of virus.

For the isolation of virus, secondary feline kidney cells were used. The cells were infected in suspension at the time of planting. An inoculum of 0.2 ml was used for 20 ml of cell suspension containing 10<sup>5</sup> cells per ml.

The cells were cultivated in glass medicine bottles with a growth surface of 130 mm<sup>2</sup> containing 20 ml of medium. Following infection, the cells were incubated for five to six days at 37°C. The harvested fluids were then tested by the haemagglutination test and used as the inoculum for the second passage following the same procedure as described above. At least two passages were carried out for each attempted isolation.

**Animal Inoculation**

**Experiment 1 – Establishment of safety of the vaccine virus.** Fluids from passages 42, 58 and 67 of the virus were administered in one to ten doses by the subcutaneous route in 1 ml amounts. One dose is defined as 1 ml of tissue culture fluid having a virus haemagglutination titre of 1:64. Five doses of passage 67 were also given orally to susceptible mink to test for virulence. All mink were observed for symptoms of mink enteritis over a 21 day period.

**Experiment 2 – Isolation of virus from inoculated mink.** As the vaccine contained a live virus an experiment was designed to determine whether live virus could be isolated from mink following inoculation. Each of ten mink was inoculated subcutaneously with ten doses of the vaccine virus. Starting from the first day after inoculation, two mink were sacrificed daily for five days and attempts to isolate the mink enteritis virus from the liver, spleen and serum were made in tissue cultures using the techniques described previously.

**Experiment 3 – Back passage studies.** It was determined from experiment 2 that the spleen harvested on the third day was a good source of virus. The vaccine virus, isolated from the spleen on the third day of experiment 2, was injected subcutaneously into two mink using 1 ml of 10% spleen suspension. After three
days the mink were sacrificed and a 10% suspension of spleens prepared. This suspension was then used for the inoculation of another two mink and this procedure was followed until six passages were completed. From the sixth passage a 10% suspension of the large intestine and a 10% suspension of spleen were prepared. Virus was isolated from both the spleen and large intestine suspensions in primary feline kidney cells. Five or six mink were then inoculated subcutaneously with 1 ml and orally by stomach tube with 5 ml of the spleen and intestinal suspensions. A virulent low passage mink enteritis virus was also given to mink by the same routes to confirm the susceptibility of the mink on test.

Experiment 4 – Shedding of vaccine virus in feces following inoculation of mink. After determining that the vaccine virus was present in mink tissues after inoculation with mink enteritis virus, a trial was designed to determine if the virus was shed in the feces. Fecal samples were collected daily for seven days from 30 mink, each of which had been inoculated with ten doses of the vaccine subcutaneously, and also from five uninoculated mink. Samples were also collected between the 14th and 16th days. An attempt to isolate the vaccine virus following three passages in secondary feline kidney cells was made on individual samples over a seven day period and successful isolations were made on individual samples during this period. Virus isolations were attempted on three pools, ten fecal samples in each, from vaccinated mink collected on the first seven days and also on days 14, 15 and 16.

Experiment 5 – Isolation of vaccine virus from mink tissues after vaccination. From ten mink inoculated with mink enteritis vaccine three months previously, an attempt was made to isolate the virus from a pool of livers, spleens and kidney tissues and from a pool of the large intestines and also from nine uninoculated contact controls. At the same time, attempts were made to isolate virus from similar tissues from vaccinated mink sacrificed ten days after a challenge with virulent mink enteritis virus. In each case, three passages were carried out as described previously, but no virus was isolated. These results suggest that there is no carrier state of the vaccine virus in the late postvaccination period.

To investigate this further, a pool of a 10% suspension of liver, spleen, kidney and large intestine was made from each of the following: ten vaccinated mink referred to in experiment 5, ten vaccinated mink sacrificed following a challenge with virulent mink enteritis virus and six mink vaccinated with an inactivated mink enteritis virus vaccine, which were subsequently challenged with virulent mink enteritis virus. Each of these preparations was then inoculated subcutaneously, in 1 ml amounts, into six mink. Three weeks after inoculation all mink, along with six unvaccinated animals, were challenged with virulent mink enteritis virus and observed for nine days for symptoms of mink enteritis.

Experiment 6 – Field trial studies. Following completion of laboratory observations, two field trials with approximately 5,000 mink in each, were carried out. The first of the two trials involved 4,916 mink which were vaccinated with avirulent mink enteritis vaccine, while in the second trial the mink enteritis vaccine was given in combination with mink distemper vaccine and Clostridium botulinum toxoid to 5,081 animals. All mink were given one dose of the vaccines subcutaneously and observed for symptoms of mink enteritis for five to six weeks after vaccination.

Experiment 7 – Horizontal spread of vaccine virus. In order to determine if the virus spread from vaccinated to nonvaccinated animals, nine unvaccinated mink from each of nine ranches which had been kept in close contact with vaccinated animals in the field trial study, were challenged with virulent mink enteritis virus, approximately three months after the study had begun. The animals were observed for nine days for symptoms of mink enteritis.

Experiment 8 – Antigenic extinction studies. Mink were vaccinated with various preparations of vaccine from undiluted through to a 10−4 dilution. None of the vaccinated mink showed any symptoms of mink enteritis during the three week postvaccination period. Following the observation period all animals, including ten unvaccinated mink, were challenged with virulent mink enteritis virus.

Experiment 9 – Combination of mink enteritis vaccine with mink distemper vaccine and Clostridium botulinum toxoid. Having demonstrated that the attenuated live mink enteritis virus was both safe and antigenic in mink, it was combined with an attenuated live mink distemper virus and with Clostridium botulinum toxoid in an attempt to immunize mink against three diseases with a single inoculation. Both the mink distemper vaccine and the Clostridium botulinum toxoid were
Experiment 1. Table I summarizes the results of the safety test carried out on the 42nd, 58th and 67th passage of the virus. Table II shows the results of a detailed safety test of the 67th passage which was carried out in susceptible weanling mink kits. Three of 68 mink receiving the 42nd virus passage showed typical symptoms of mink enteritis, indicating that the virus had not been completely attenuated. However, virus from the 58th and 67th passages caused no clinical symptoms of mink enteritis.

**TABLE I**

**RESULTS OF ADMINISTERING THE 42ND, 58TH AND 67TH TISSUE CULTURE PASSAGE OF THE MINK ENTERITIS VIRUS TO SUSCEPTIBLE MINK**

<table>
<thead>
<tr>
<th>Virus Passage</th>
<th>Dosage</th>
<th>Route</th>
<th>Positive/Total&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>1</td>
<td>s/c&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/68</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>s/c</td>
<td>3/68</td>
</tr>
<tr>
<td>58</td>
<td>10</td>
<td>s/c</td>
<td>0/71</td>
</tr>
<tr>
<td>67</td>
<td>1</td>
<td>s/c</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>oral</td>
<td>0/5</td>
</tr>
<tr>
<td>Contact Controls</td>
<td></td>
<td></td>
<td>0/73</td>
</tr>
</tbody>
</table>

<sup>a</sup>s/c = subcutaneous.
<sup>b</sup>Clinical observations 21 days postvaccination.

**TABLE II**

**RESULTS OF ADMINISTERING THE 67TH PASSAGE OF MINK ENTERITIS VIRUS TO WEANLING MINK**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Route</th>
<th>Mink Inoculated With</th>
<th>Positive/Total&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>s/c&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Vaccine virus</td>
<td>0/10</td>
</tr>
<tr>
<td>10</td>
<td>s/c</td>
<td>Vaccine virus</td>
<td>0/54</td>
</tr>
<tr>
<td>5</td>
<td>oral</td>
<td>Vaccine virus</td>
<td>0/10</td>
</tr>
<tr>
<td>Contact Controls</td>
<td>No virus</td>
<td></td>
<td>0/20</td>
</tr>
<tr>
<td>Challenge Controls</td>
<td>Virulent mink enteritis virus</td>
<td></td>
<td>4/4</td>
</tr>
</tbody>
</table>

<sup>a</sup>s/c = subcutaneously.
<sup>b</sup>Clinical observation 21 days postvaccination.

previously shown to be safe and antigenic when tested as single components. In order to demonstrate that all three antigens could be combined effectively into a single product, a trial was designed in which the distemper and enteritis vaccine viruses were pooled and lyophilized. The combined lyophilized vaccine was reconstituted with *Clostridium botulinum* toxoid. After one hour at room temperature the reconstituted vaccine was diluted to 1:6 and 1:60. Each of these preparations was administered subcutaneously to mink in 1 ml amounts. Three weeks after vaccination the vaccinated mink and six unvaccinated animals were challenged with *Clostridium botulinum* toxin. Five days following the botulinum challenge, all surviving animals in the vaccinated group were challenged, along with an additional six unvaccinated mink, with virulent mink enteritis virus. Following the challenge with mink enteritis virus, all surviving vaccinated animals and six unvaccinated animals were challenged with mink distemper virus.

**RESULTS**

*Experiment 1.*
ENTERITIS VACCINE

TABLE IV

RESULTS OF INOCULATING THE SIXTH, BACK-PASSAGE ATTENUATED, MINK ENTERITIS VIRUS AND VIRULENT, LOW PASSAGE VIRUS INTO MINK

<table>
<thead>
<tr>
<th>Source of Virus</th>
<th>Route</th>
<th>Dosage (ml)</th>
<th>Clinical Picture 8 Days Postvaccination Positive/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>s/c</td>
<td>1</td>
<td>0/5</td>
</tr>
<tr>
<td>Intestine</td>
<td>oral</td>
<td>5</td>
<td>0/6</td>
</tr>
<tr>
<td>Spleen</td>
<td>s/c</td>
<td>1</td>
<td>0/5</td>
</tr>
<tr>
<td>Spleen</td>
<td>oral</td>
<td>5</td>
<td>0/5</td>
</tr>
<tr>
<td>Virulent low passage virus</td>
<td>s/c</td>
<td>1</td>
<td>4/6</td>
</tr>
<tr>
<td>Virulent low passage virus</td>
<td>oral</td>
<td>5</td>
<td>4/6</td>
</tr>
</tbody>
</table>

enteritis even when multiple doses were administered by the subcutaneous or oral route.

Experiment 2. Table III shows the results obtained on isolation of virus from various tissues of inoculated mink. The results indicate that the vaccine virus can be found in various tissues over a five day period following inoculation. The virus was found in all the tissues tested on the third day. The spleen appeared to be the most consistent source of virus.

Experiment 3. The results of inoculating mink with the sixth back-passage of the attenuated mink enteritis virus and a low passage virulent virus are summarized in Table IV. Neither suspension of the sixth back-passaged material caused symptoms of enteritis when given either by the subcutaneous or oral route. In contrast the low passage virulent mink enteritis virus caused clinical symptoms when administered by both routes. It would appear that the avirulent vaccine virus is stable and does not revert to virulence following six serial passages in susceptible mink.

Experiment 4. Table V shows the results obtained on the isolation of virus from the feces of the 30 vaccinated mink over a seven day period, whereas Table VI shows a summary of these results along with the five unvaccinated mink. Virus was isolated from 21 of the 30 vaccinated mink at one time or another over the seven day period. The highest number of isolations was obtained on day five after which isolations declined sharply. The results obtained on isolation of virus from the pooled samples are shown in Table VII. No virus was found in the pooled fecal samples after day 6 although contamination may have masked the isolation of virus in the seven day sample.

Experiment 5. When the suspensions of mink tissues, as described in this trial, were inoculated into susceptible mink, no clinical symp-
Experiment 6. In the two field trials described, a total of 9,997 mink were vaccinated with the attenuated mink enteritis virus. None of these animals showed clinical symptoms of enteritis over a five week observation period, as shown in the summary in Table IX.

Experiment 7. In this trial the unvaccinated mink, which had been left in close contact with vaccinated mink for a three month period, were susceptible on challenge with virulent mink enteritis virus as can be seen in Table X. This susceptibility demonstrates a lack of spread of the vaccine virus from vaccinated to unvaccinated animals under field conditions.

Experiment 8. All mink, up to and including the 10⁻³ dilution of the vaccine were protected fully against the challenge, whereas 73% of the unvaccinated mink developed symptoms of the disease (Table XI). This demonstrates that the vaccine virus is highly immunogenic.

### Table VI

<table>
<thead>
<tr>
<th>Day Post-vaccination</th>
<th>Vaccinates</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Positive*/Total</td>
<td>% Positive</td>
</tr>
<tr>
<td>1</td>
<td>8/28</td>
<td>28.5</td>
</tr>
<tr>
<td>2</td>
<td>7/27</td>
<td>25.9</td>
</tr>
<tr>
<td>3</td>
<td>12/29</td>
<td>41.3</td>
</tr>
<tr>
<td>4</td>
<td>4/30</td>
<td>13.3</td>
</tr>
<tr>
<td>5</td>
<td>13/30</td>
<td>43.3</td>
</tr>
<tr>
<td>6</td>
<td>3/30</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>2/30</td>
<td>6.6</td>
</tr>
</tbody>
</table>

*Virus isolation.

stance such as would occur if residual virus had been present.

Experiment 9. The results in Table XII suggest that all three vaccine components of the vaccine are highly protective even when diluted. There was complete immunity in the vaccinated mink to both distemper and botulinum with the 1:60 dilution of vaccine against a challenge which caused 100% of the controls to show symptoms. Against enteritis, a 67% protection was provided by the vaccine diluted to 1:60 and with vaccine diluted 1:6, 100% of the mink were protected against a strong challenge which caused enteritis in all of the nonvaccinated controls.

### Discussion

A virulent mink enteritis virus was attenuated after 67 passages in feline kidney cell culture. At this level of passage the attenuated virus appeared to be stable as shown by failure to revert to virulence after six serial back-passages in susceptible mink.

The vaccine was shown to be safe even when multiple doses were inoculated into very young mink. The safety of the vaccine was confirmed by carrying out two field trial experiments in which 4,916 and 5,081 mink respectively were vaccinated, under field conditions, with no symptoms of mink enteritis appearing. In one trial, unvaccinated mink, which were held in close contact with vaccinated mink, were susceptible to challenge with virulent mink enteritis virus. This indicates that the vaccine virus did not spread readily from vaccinated to unvaccinated animals.

Although the vaccine virus was found in the feces of vaccinated mink, the shedding was limited to the early postinoculation period. No virus was isolated from samples of pooled feces of vaccinated mink from the 14th to 16th day. Although only pooled samples were tested, it should be noted, when comparing

### Table VII

<table>
<thead>
<tr>
<th>Pool No.</th>
<th>Days Postinoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Pool #1 (Mink 261–270)</td>
<td>+</td>
</tr>
<tr>
<td>Pool #2 (Mink 271–280)</td>
<td>+</td>
</tr>
<tr>
<td>Pool #3 (Mink 281–290)</td>
<td>+</td>
</tr>
</tbody>
</table>

Symbols Used: + positive HA = Virus present; – Negative HA = Virus absent; C Contaminated sample.
the results of individual isolations from feces collected during the first seven days after vaccination with those isolations made from the pooled samples, that in five of six cases the virus was isolated from the pooled sample when only one mink in the pool was shedding virus. The vaccine virus was found in the tissues of mink immediately following inoculation. Virus could not be isolated however, from any of the tissues tested three months after vaccination nor did these tissues contain any immunizing substance when inoculated into mink. These findings are not in agreement with those of Bouillant and Hanson (2). There were, however, differences in experimental design that could account for the discrepancy. Our experiments showed no evidence that the vaccine virus was shed after the early postvaccinational period and our methods could not detect any carrier animals.

The attenuated virus provided a good, immunogenic response in mink whether used alone or when used in combination with other antigens. The results obtained on challenge when the distemper and enteritis vaccines were combined with Clostridium botulinum toxoid indicated that such a combination could be used to protect mink against all three diseases by a single injection.

**SUMMARY**

An attenuated live virus mink enteritis vaccine was developed by serial propagation of a virulent virus in feline kidney tissue cultures. Safety of the vaccine virus was demon-
strated after 67 passages. It was shown that the vaccine virus was stable and did not revert to virulence even after six serial back-passages in mink. Although the virus can be isolated from tissues and feces of mink for several days following vaccination, the virus did not spread from vaccinated to nonvaccinated animals kept in close contact over a three month period. The vaccine showed a good immunogenic response in mink when used either alone or in combination with mink distemper vaccine and Clostridium botulinum toxoid, type C, beta.

RÉSUMÉ
Les auteurs ont développé un vaccin atténué contre l'entérite à virus du vison, en effectuant plusieurs passages successifs d'une souche virulente de ce virus, sur des cultures de cellules rénales félines. Ils démontrèrent l'innocuité du virus du vaccin, après 67 passages; ils en démontrèrent aussi la stabilité et l'absence d'un retour à la virulence, même après six passages successifs, chez des visons. Bien que l'isolement du virus se soit avéré possible à partir des tissus et des fèces des sujets vaccinés, pour plusieurs jours après la vaccination, il ne se propagea pas aux visons témoins qui restèrent en contact étroit avec les sujets vaccinés, durant une période de trois mois. L'utilisation de ce vaccin, seul ou avec le vaccin contre le distemper du vison et la toxoïde de Clostridium botulinum, type C, béta, suscita une bonne immunité.

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REFERENCES

INVITATION À DES FONDS DE RECHERCHE
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