Preparation of Fowl Pox Vaccine on Chicken-Embryo-Dermis Cell Culture

A. El-Zein, S. Nehme, V. Ghorraib, S. Hasbani, and B. Toth
Department of Virology, Regional Poultry Laboratory Research Institute of Agriculture, Fanar, Lebanon

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SUMMARY

A chicken-embryo-dermis (CED) cell-culture system derived from the skin of a specific-pathogen-free embryo is defined and shown to be highly susceptible to the fowl pox virus (FPV). The high susceptibility of the system and the growth characteristics of the virus suggest that the host-tissue specificity for FPV persists after the cells have differentiated in cell cultures. The cell system consists of cell strains derived from primary CED cell cultures. A vaccinal strain of FPV of Canadian origin (CFPV) was adapted to the cell system, and an experimental batch of vaccine was prepared from the 5th passage and tested. The cell-culture vaccine has higher biological properties than a conventional CFPV vaccine prepared on the chorioallantoic membrane of embryonated eggs. The advantages of the cell-culture FPV vaccine are discussed. The behavior and requirements of the CED cell system are described.

INTRODUCTION

Several investigators have reported the growth of fowl pox virus (FPV) in various cell-culture systems (2,3,7,9,10,13) and possible use of some of these host systems for vaccine preparation (8,12). Common to those reports are the relatively low virus titers obtained and the absence of intracytoplasmic inclusion bodies (2, 4,5) in the infected cell cultures. When FPV is grown in a susceptible in vivo system or in vitro organ culture, however, it yields a higher virus titer and regularly causes the formation of specific intracytoplasmic inclusion bodies. The latter appear to play a role in maturation of the FPV by providing it with an outer layer of lipoproteins (1), thus explaining the relations between their

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^Present address: Director, Phylaxia Institute, Veterinary Biologials, Budapest, Hungary.
presence and the high virus titer. Conversely, the low virus titer and lack of inclusion formation suggests that the cell-culture systems reported were of limited susceptibility to the FPV.

Since the FPV is dermotropic, exerting its pathological activity within the skin tissues, it was thought that a cell-culture system derived from this target organ might retain the host-tissue specificity for the virus and provide a highly susceptible in vitro cell system.

This paper describes a chicken embryo dermis (CED) cell-culture system which has proved to yield a high virus titer and support the formation of inclusion bodies. Also described are the use and advantages of this cell system for the production of fowl pox vaccine.

MATERIALS AND METHODS

Virus strains. An egg-adapted fowl pox vaccinal strain was obtained from the Phylaxia Institute, Budapest, Hungary, and used for adaptation and preparation of the experimental batch of vaccine on CED cell culture. The strain originated from Canada and has been used since 1970 in the Phylaxia Institute for the production of fowl pox vaccine by the conventional method of virus propagation on the chorioallantoic membrane (CAM) of embryonated eggs. The strain had undergone an unknown number of passages on CAM since its original isolation in Canada. It was passaged twice in our laboratory on the CAM of specific-pathogen-free (SPF) eggs, and a lyophilized virus stock was prepared from the infected CAM of the 2nd egg passage (SPF fertile eggs were obtained from Lohmann & Co., Cuxhaven, West Germany). The virus stock was titrated on the CAM, with the end point calculated as the 50%-egg-infective dose (EID$_{50}$) by the Reed and Muench method (11). The titer was $10^{7.7}$ EID$_{50}$ per ml. The strain proved highly pathogenic to embryos, systematically causing a hemorrhagic type of pocks on the CAM of embryonated eggs. Pathogenicity was also expressed as a 50%-embryo-lethal dose (ELD$_{50}$) and was of the order of $10^{4.83}$ ELD$_{50}$ per ml. In experimental chickens, skin scarification or wing-web stick with undiluted preparations of the strain was limited to local reactions at the sites of inoculation.

Used for challenge was a Lebanese fowl pox isolate (LFP) that had undergone 5 to 8 passages on the CAM of embryonated eggs since its initial isolation. Its pathogenicity to chicken embryos was lower in that it caused the formation of clear whitish pocks on the
Preparing fowl pox vaccine

CAM and had an ELD$_{50}$ titer of $10^{2.86}$ per ml. The LFP was more pathogenic to susceptible chickens, however, since it caused severe local reaction around the inoculation site plus a general loss of condition. The LFP also caused an occasional generalized spread over the head with $10^{-1}$ and $10^{-2}$ dilutions of the virus preparation.

Cell cultures. Primary CED was prepared by removing aseptically the skin of 12-day-old SPF embryos. These skins were cut into small pieces with scissors and washed 3 times with Puck's saline-A solution. They were then digested several times with a 0.05% trypsin (Difco 1/250) solution prepared in Puck's saline-A for 5 minutes each time. The trypsin solution was preheated to 37 C, and trypsinization was done with a magnetic stirrer. The first two trypsinizations were discarded, and the following ones were collected individually in an equal volume of refrigerated saline-A solution containing 20% calf serum (CS) and subsequently stored in an ice bath. The collected cell suspensions were pooled, filtered through two layers of gauze, and sedimented at 1000 rpm for 5 minutes. After the supernatant fluid had been discarded, the cells were resuspended in medium 199 with Earle's salt supplemented by 10% tryptose phosphate and 5% CS. The supplemented medium contained either a combination of 100 I.U. of penicillin and 0.1 mg of streptomycin, or 0.05 mg of gentamycin (Scherring Co.) per ml. The final cell-suspension preparation contained $6 \times 10^5$ cells per ml. The cells were grown in test tubes (16 × 150 mm), and each tube was seeded with 1 ml of cell suspension. Monolayers were obtained 48 hours after seeding.

Cell strains were derived from the primary cultures by serial transfers. The primary cell suspensions were grown in 2-ounce prescription bottles, each bottle being seeded with 5 ml of cell suspension. Two to 3 days later the cells were forming complete monolayers. The cell sheets were then treated with a solution of 0.05% trypsin and 0.02% versene prepared in Puck's saline-A preheated at 37 C. The cell harvest was transferred in a proportion of 1:3, resulting in a final suspension of about $2 \times 10^5$ cells per ml.

The experimental batch of vaccine was prepared on cell sheets grown in Roux flasks. Each flask was seeded with 100 ml of cell suspension. The cells were used after their 3rd passage.

All cultures were closed with rubber stoppers and incubated at 37 C in a stationary position.
**Virus titration.** Susceptible 6-week-old Leghorn chickens were used for virus titration. The virus materials were diluted into 10-fold serial dilutions (10⁻¹ to 10⁻⁹). Five chickens were used for each virus titration, and each chicken was inoculated with the 6 virus dilutions by means of skin scarification at 6 different sites on the two legs. Each bird was also inoculated by the wing-web stick method with the same virus dilutions used at different sites on the two wings.

To estimate the degree of pathogenicity of the two virus strains in birds as expressed by the degree of local reactions and the possible generalization, the titration was carried out as follows: Two birds were used for each dilution and each dilution was inoculated by scarification of the legs and also by the wing-web stick technique. Twelve chickens were used for each virus titration.

Titration on the CAM was done with virus dilutions from 10⁻¹ to 10⁻⁹. Each dilution was inoculated via the artificial air cell into 5 SPF eggs 11 days old, 0.2 ml per egg, by the Cunningham method (6). The eggs were candled each day for 5 days. Deaths in the first 24 hours after inoculation were discounted, and the remaining were recorded for estimation of the ELD₅₀. All the eggs were opened at the 5th day postinoculation, and the EID₅₀ was estimated by the

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**Fig. 1.** Morphological aspect of fibroblasts of CED culture at the 4th cell passage.
presence or absence of the specific pox reaction. The types of pocks were observed at the high dilutions, but they were not always counted.

Titration of the Canadian fowl pox virus (CFPV) strain in cell culture was done by inoculating 5 CED cell-culture tubes with 0.1 ml per tube. The virus was adsorbed at 37 °C for 2 hours, after which 1 ml of 199 medium containing 2 to 3% CS was pipetted into each tube. The cultures were examined for cytopathic effect (CPE) for 7 days, after which the 50% tissue-culture-infective dose (TCID₅₀) was estimated.

**Preparation and testing of CFPV cell-culture vaccine.** The 4th passage of the CFPV was inoculated into 5 Roux flasks containing monolayers of CED cell cultures at their 3rd passage. Each flask was inoculated with 5 ml of a 1/200 virus dilution. The virus was adsorbed for 2 hours, after which 100 ml of maintenance medium was added to each flask. The flasks were incubated for 4 to 6 days, by which time the CPE was advanced. They were then transferred to -20 °C, and the cells with the infected medium were frozen and thawed twice. The fluids containing the broken infected cells were collected, pooled, homogenized with a magnetic stirrer, and stored at -20 °C. A volume of 5 ml was taken from the homogenized pool for titration to estimate the vaccinal dose and the proper factor to end up with a final product containing 1000 vaccinal doses per ml.

The vaccinal dose was estimated as $3 \times 10^4$ EID₅₀ or $3 \times 10^{4.5}$ TCID₅₀, i.e., each ml of cell-culture virus preparation corresponded to 3000 vaccinal doses. The vaccinal dose thus calculated was further tested to give 100% take at 1/10 dilution upon vaccination of 20 susceptible birds by the stick-web method.

Also tested was a conventional vaccine prepared on CAM containing $3 \times 10^4$ EID₅₀ per vaccinal dose.

Two series, each of 20 birds, were vaccinated separately with the cell-culture and the CAM vaccines. Three weeks later they were challenged with a $10^{-1}$ dilution of the LFPV by skin scarification on the leg. Fifteen unvaccinated birds of the same age were inoculated with the same challenge dose and kept as controls.

**RESULTS**

**Behavior of CED cell cultures.** The cellular morphology of the primary CED cultures was of a mixed type. The majority of the cell population was of the fibroblastic type, and the remainder was epithelial. After monolayer formation the epithelial cells were
grouped into colonies which had diffused edges where epithelial and fibroblastic cells were intermixed. At the 3rd or the 4th passage, the cells were uniformly of fibroblastic morphology, being spindle-shaped, and in areas of high cell density, were oriented with their long axes parallel to one another (Fig. 1).

A limiting factor for cell growth was the percentage of CS in the culture medium. Five percent CS was the optimal serum concentration that ensured a high rate of cellular replication. A percentage of 8 to 10% CS proved toxic for the cells. Cells which were forming monolayers showed a rounding-up and massive detachment from the glass surface 24 hours after being fed with medium containing 10% CS. Cells seeded with such medium grew well during the first 16 to 24 hours but later degenerated without reaching the stage of monolayer formation. A percentage of 2 to 3% CS was not high enough for active growth, but maintained the cell monolayers in good state up to 14 to 16 days with changes of medium every 48 hours.

The CED cells were passaged frequently (5 to 6 passages) with an excellent rate of replication. From the 6th passage onward, growth was slower. The maximum number of passages obtained under experimental conditions was 9 passages. The following pas-
sage showed a bizarre type of cell unable to reach the stage of monolayer formation.

The maximum cellular yield per skin of each 12-day embryo was 30 ml of cell suspension containing $6 \times 10^6$ cells per ml. A mean of 25 ml of cell suspension per skin of embryo was usually obtained.

**Adaptation of the CFPV to CED cell culture.** The first virus passage on CED cell culture produced clear CPE the 4th day after inoculation. CPE was complete 2 days later. On the following virus passages, CPE was clear 24 hours after inoculation and was complete 2 days later.

Both types of cell (fibroblastic and epithelial) were equally susceptible to the virus as judged by the formation of intracytoplasmic inclusions (Figs. 3, 4). The inclusions were clear and easy to locate 36 to 48 hours postinfection. About 30 to 35% of the infected cells harbored inclusions. A good percentage of the fibroblastic cells had bipolar inclusions, as seen in Fig. 4. By the 72nd hour the inclusions were rare and most of the cells were showing degenerated cytoplasm with huge vacuoles, some containing an agglomerate of small filaments. Large inclusions associated with the above-described vacuoles (Fig. 5) were seen in 3 to 4 cells of one coverslip preparation of infected cells.

![Fig. 3. Intracytoplasmic inclusion bodies in an epithelial cell of CED culture infected with CFPV 48 hours after infection.](image-url)
Table 1. Comparative titration of CFPV prepared on CED cell culture and on CAM of embryonated eggs.

<table>
<thead>
<tr>
<th>Titration procedure</th>
<th>Log titer of CC vaccine&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Log titer of CAM vaccine&lt;sup&gt;B&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>8.5/ml</td>
<td>ND&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>EID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>8.0/ml</td>
<td>8.3/ml</td>
</tr>
<tr>
<td>CID&lt;sub&gt;50&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>— wing web stick</td>
<td>5.3/ml</td>
<td>4.5/ml</td>
</tr>
<tr>
<td>— scarification</td>
<td>6.9</td>
<td>4.3</td>
</tr>
</tbody>
</table>

<sup>A</sup>Cell-culture vaccine  
<sup>B</sup>Not done

The yield of the CFPV on primary cell cultures was $10^{8.5}$ TCID<sub>50</sub> per ml. Repeated titrations of the same virus preparation on tertiary and on the 5th passage of CED cell strain respectively revealed mean titers of $10^{8.5}$ and $10^{8.3}$.

Comparative titration of CFPV prepared on CED cell culture and on CAM of embryonated eggs. Table 1 contains the results of this comparative titration. The titers of the two vaccines were comparable by EID<sub>50</sub> but by 50%-chicken-infective dose (CID<sub>50</sub>) were significantly higher with cell-culture vaccine preparations.

**Results of challenge.** Reactions were mild at the site of inoculation after challenge in both groups vaccinated with CAM and cell-culture vaccines. The reaction started on the third day after challenge as a hyperplasia of the epidermis and the feather follicles. The

Fig. 4. Bipolar intracytoplasmic inclusion bodies in a fibroblast cell of CED culture infected with CFPV 48 hours after infection.
lesions were more pronounced 24 hours later. In the 5th day after challenge the reaction had completely regressed to the normal state in the cell-culture-vaccinated group. In the CAM-vaccinated group the regression was gradual from the 5th to the 8th day after challenge. In both groups the feathers started to grow normally at the site of inoculation at the beginning of the third week after challenge. No loss of condition was observed in either group.

The local reaction in the control group started on the 3rd day after inoculation, with hyperplasia in the feather follicles and marked congestion in and around the inoculated area. The lesions progressed from the 5th to the 8th day, when they reached their maximum with petechial formation at the base of the affected follicles and the formation of nodules which first appeared as small white foci which grew rapidly and became yellow. By the 8th day after inoculation the congestion had regressed but the majority of the affected follicles became hemorrhagic and the adjoining nodules had coalesced and become rough and dark-brown areas. Scabs formed by the end of the second week; a good number of them dropped off naturally by the end of the third week, leaving smooth scars, whereas the remainder persisted, with a seropurulent exudate at the edges of the scabs. The birds were kept for 35 days after

Fig. 5. Huge vacuoles associated with big inclusions in CED culture infected with CFPV. Note the granulated filaments within one vacuole. 72 hours after infection.
challenge with no indication of feather growth at the site of inoculation. The general conditions of the birds were severely affected during the 2nd and 3rd weeks after inoculation. Two birds showed only generalized lesions on the head at the 3rd week after challenge.

**DISCUSSION**

The CED cell-culture system seems to be the system of choice for the *in vitro* growth of FPV. The cells are easy to grow and can be maintained for long periods (14 to 18 days). The only limiting factors for cellular growth are serum toxicity and trypsin sensitivity. Concentrations of serum higher than 5% in the culture medium are cytotoxic to the system; conversely, trypsinization with the usual 0.25% trypsin concentration affects the cells irreversibly. Trypsinization trials with this usual concentration for varying periods failed to give satisfactory cellular growth in primary cultures.

The CED is a highly economical system for production of fowl pox vaccine because the virus yields are high: 1 ml of virus cell-culture preparation has yielded 3000 doses of vaccine with each dose containing approximately $3 \times 10^{4.5}$ TCID$_{50}$. It was estimated that cell cultures derived from the skin of 8 embryos and transferred through 4 passages would yield, after virus infection, $12 \times 10^6$ vaccinal doses. The same quantity of vaccine produced by the conventional method of virus growth on the CAM of embryonated eggs would require 3600 eggs, obviously more expensive and much more time-consuming. Since fowl pox vaccine is used to vaccinate breeders and layers, SPF eggs are recommended for preparation, making the procedure even more expensive.

Not only is CED cell culture less expensive than the CE host system but the vaccine produced has the common biological advantages of a cell-culture vaccine: homogeneity, uniformity, and strict bacterial and fungal sterility in the final product. The significantly higher CID$_{50}$ titer with the CED cell-culture vaccine might be the result of homogeneous free virus particle distribution in the preparation. Preparation from ground infected CAM, in contrast, contains a high percentage of clumped particles, which alters the distribution among the inocula and might affect the diffusibility of the virus through the skin. In addition, the CED cell-culture vaccine has a higher virus titer per unit volume than those of other proposed FPV cell-culture vaccines, which permits preparation
of potent vaccine with a high virus titer per vaccinal dose. Gelenczei and Lasher (6) reported a log titer of 5.2 EID₅₀ per ml in whole duck-embryo cell culture, and Doyle et al. (7) reported a log titer between 4 and 5 TCID₅₀ per ml in whole chicken-embryo cultures. Such titers are close to the minimum requirement per ml for a fowl pox vaccine product needed for immune response in chickens as stated by Winterfield and Hitchner (14), whereas the CED cell-culture system commonly gave a log virus titer of 8.5 TCID₅₀ per ml.

The optimal embryo age used for CED cell culture was 12 days old. It was selected because of early feather appearance during embryonic life. At 14 days old the feathers are complete and toxic for the cultured cells. At 13 days old about 40% of the embryos have feathers. Also, at 12 days old the skin is devoid of feathers and is easily removed. It is possible to derive cultures from the skin of 7-to-11-day-old embryos, but the skin proved difficult to remove from the body and gave a lower cellular yield; furthermore, there were no advantages as far as cellular growth rate was concerned.

The stability of the virus titer on primary, tertiary, and 5th passages of the CED cell strains indicated that the cells had retained their susceptibility during this limited number of passages. It adds further evidence that both cellular types, epithelial and fibroblastic, are equally susceptible to FPV, since the loss of epithelial cell population around the 3rd cell passage did not affect the titer.

The cellular susceptibility of the CED cell system to FPV remains the most interesting feature in this investigation. The fact that this susceptibility seemed to be related to the presence of inclusion bodies may clarify the role of these inclusions in the process of virus maturation. Arhelger and Randall (1) have shown evidence that the inclusion bodies provide FPV particles with an outer layer which may be lipidic or lipoproteinic in nature. Conversely, the absence of inclusions in chicken epithelial cell culture (2) and in the short-term culture of chicken embryo (4) associated with low virus titers in these cell systems (2,7,8) adds yet more evidence of the role of inclusion bodies in the process of virus replication and maturation.

To confirm that the inclusion bodies are related to cellular factors, not to virus strain factors, the CFPV was grown in whole chicken embryo (WCE) and chicken embryo kidney (CEK) cell culture. Typical fowl pox CPE were observed in both cell systems.
and were much more severe in CEK than in WCE, but no inclusion bodies were formed in either cell system.

The relationship between inclusion bodies and cellular susceptibility as measured by higher virus yield would substantiate the possibility of host and tissue specificities for virus infection to persist in an experimental in vitro cellular system when cells are derived from the relevant tissue.

REFERENCES