Vaccines and Related Biological Products Advisory Committee (VRBPAC)

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Madin Darby Canine Kidney Continuous Cell Line

Briefing Document

Solvay Pharmaceuticals

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1.0 EXECUTIVE SUMMARY

Production of influenza vaccines requires propagation of large quantities of the specified virus, which requires the use of a live cell substrate. Historically, influenza vaccine strains have been propagated in embryonated chicken eggs. Continuous cell lines (CCLs) such as Madin Darby canine kidney (MDCK), Vero (African green monkey kidney), and PER.C6 (human fetal retina) provide alternatives to embryonated chicken eggs for use as substrates. In contrast to the use of embryonated eggs, cell-based manufacturing allows for a better-controlled substrate and a closed production process utilizing bioreactors. Egg-based influenza vaccine production is dependent on the availability of embryonated eggs, which is at risk in the event of outbreaks of bird diseases. In addition, use of a continuous cell line will allow manufacturers to respond more quickly to the emergence of new strains of influenza in a pandemic situation. Establishment of cell-based influenza vaccine production methods is supported by the National Institutes of Health (NIH), World Health Organization (WHO), and Centers for Disease Control and Prevention (CDC).

Changing from egg-based to cell-based vaccine production requires a thorough investigation of the starting materials and the steps to produce cell derived–inactivated subunit influenza vaccine (CD-ISIV). Solvay Pharmaceuticals has manufactured and marketed an egg-derived subunit influenza vaccine, tradename Influvac®, for over 50 years. This egg-based vaccine is approved and marketed in 56 countries worldwide. For over 10 years, Solvay Pharmaceuticals has been conducting research and development on novel influenza delivery and production technologies, in particular cell-based substrates for the production of influenza vaccine. After a rigorous evaluation of the cell-based substrate options, Solvay Pharmaceuticals sought to further develop the MDCK cell line for its superior growth characteristics for the broadest spectrum of influenza viruses. MDCK cells are also employed by most surveillance laboratories worldwide. Because MDCK cells are of canine origin, the risk that they contain adventitious infectious agents with affinity to humans is lower than with cells of human or other primate origin. The use of MDCK cells does not raise concerns about diseases like human immunodeficiency virus (HIV) or bovine spongiform encephalopathy (BSE) agents. Furthermore, MDCK cells are not derived from a source that is subject to ethical debate. To support the use of the MDCK CCL for influenza vaccine production, Solvay Pharmaceuticals has conducted a risk assessment specific to this substrate.

Solvay Pharmaceuticals purchased the MDCK cell line (passage 52) from the American Type Culture Collection (ATCC) and established well-documented master and working cell bank stocks (passages 56 and 57, respectively). The cell line's physical characteristics (morphology and phenotype) were found to be as expected in comparison with the reference ATCC strain. Testing of cells from Solvay Pharmaceuticals' cell bank for known adventitious agents gave negative results. All media used in the process are from certified sources. Therefore, in regards to all known issues based on the best science available, Solvay Pharmaceuticals' MDCK CCL poses minimum, if any, risk for use as an influenza vaccine substrate.

Evaluation of the tumorigenic and oncogenic potential of intact MDCK cells and cellular components were conducted. Whole intact cells from Solvay Pharmaceuticals' MDCK cell line injected into immune-compromised test animals have been shown to have moderate

tumorigenic potential. At a dose level of 10^5 cells, approximately 50% of immune-compromised mice developed nodules at the inoculation site. In the majority of the animals, nodules showed complete or partial regression during a six-month observation period. Regression of the nodules was also observed in the majority of animals inoculated with 10^7 intact cells. Compared with the ATCC reference strain, Solvay Pharmaceuticals' MDCK cell line has an equivalent or slightly higher tumorigenic potential, which is likely the consequence of selection of a cell lineage with favorable growth characteristics in a serum-free medium. Neither homogenized MDCK cells nor the purified MDCK DNA fraction of the cellular extract have shown a detectable tumorigenic potential when administered by injection to various animal species. The details of all testing to date on Solvay Pharmaceuticals' MDCK cell substrate are contained within our Drug Master File (DMF) or DMF amendments to be submitted shortly.

The use of an established and characterized cell line such as Solvay Pharmaceuticals' MDCK allows for a closed, well-controlled, sterile production process with full in-process testing. Through Solvay Pharmaceuticals' manufacturing process and downstream processing, intact cells and cellular components, including DNA, are inactivated and/or removed from the final product to under 10 ng of residual DNA, thereby further reducing risk. The final product is a highly purified, thimerosal-free subunit vaccine. Solvay Pharmaceuticals is currently in the process of commercial scale-up at our plant in Weesp, the Netherlands. Process validation and batch release testing are scheduled for completion in the first quarter of 2006.

The characterization of Solvay Pharmaceuticals' MDCK CCL as a cell substrate for the production of influenza vaccine has involved comprehensive testing for adventitious agents and assessment of the risks associated with intact cells, cellular components, and cellular DNA. Based on our characterization of our MDCK cell line and the purification procedures during downstream processing, we conclude that Solvay Pharmaceuticals' MDCK continuous cell line provides a safe, well-controlled, viable alternative substrate for producing purified, inactivated subunit influenza vaccine.

2.0 INTRODUCTION

The estimated incremental healthcare burden of an influenza pandemic in the United States is staggering — an estimated 89,000 to 207,000 deaths and 314,000 to 733,000 hospitalizations [[i]]. Recent avian flu activity in southeast Asia and the increasing time since the last pandemic are believed to be indicators that another pandemic is imminent.

Influenza is the leading cause of vaccine-preventable deaths in the United States [[ii]]. Apart from the imminent threat of a pandemic, the CDC estimates that 36,000 respiratory deaths and 51,203 "all cause" deaths result from influenza annually[[iii]]. Influenza poses a significant health risk every year.

During an interpandemic year, full implementation of the CDC Advisory Committee on Immunization Practices (ACIP) current recommendation for influenza vaccine would require 185 to 190 million doses of trivalent influenza vaccine. In the event of a pandemic, the United States would need 600 million doses of monovalent pandemic influenza vaccine (2

per citizen: primer and booster). Yet over the last three years (2002 to 2004), only 85, 80, and 60 million U.S.-approved vaccine doses were produced for the U.S. market, far short of market demand and ACIP guidance. The current U.S. influenza vaccine supply is tenuous in an interpandemic season and would be inadequate in the event of a pandemic.

Furthermore, providing surge capacity via egg-based production methods is difficult if not impossible, for several reasons, starting with the logistical issues of obtaining enough embryonated eggs on short notice. Supply of embryonated eggs can be affected by environmental factors, such as circulating avian flu. Work with non-attenuated pandemic influenza strains requires higher containment, which would be difficult to achieve in egg-based production facilities. Moreover, pandemic influenza virus strains will likely originate from an avian virus and consequently be pathogenic to embryonated eggs, making egg-based vaccine production impossible. Attenuated pandemic influenza strains may be made available via reverse-genetics methodologies, but provisions must be made for potential operation at biosafety level 3 to ensure the ability to work with future pandemic viruses even if an attenuated reverse-genetics seed virus is not available.

Establishment of cell-based influenza vaccine production methods is supported by NIH, WHO, and CDC.

Solvay Pharmaceuticals is a research-based pharmaceutical company headquartered in Brussels, Belgium, with research and development and manufacturing sites in Marietta, Georgia; Weesp, the Netherlands; and Hannover, Germany. Solvay is fully integrated across all functions and is active in the therapeutic areas of cardiology, gastroenterology, mental health, women's and men's health, and specialty markets, including influenza vaccines.

Solvay has manufactured and marketed an egg-derived subunit influenza vaccine, tradename Influvac®, for more than 50 years. This egg-based vaccine is approved and marketed in 56 countries worldwide. For over 10 years, Solvay Pharmaceuticals has been conducting research and development on novel influenza delivery and production technologies, in particular cell-based substrates for the production of influenza vaccine. Our approach to development of the MDCK CD-ISIV (also known as Influvac® TC in Europe) was to establish equivalence with the egg-derived Influvac® vaccine in terms of immunogenicity and safety. The similarity of egg- and cell-derived antigens was first investigated in preclinical studies. Between 1994 and 1998, comparative clinical trials included 1,023 subjects vaccinated with CD-ISIV produced in a pilot manufacturing site. These studies demonstrated comparable safety and immunogenicity profiles between the two products. Based on these studies, Influvac® TC was granted a marketing authorization in The Netherlands in 2001. Solvay is in the process of constructing a dedicated manufacturing site for cell-derived influenza vaccine in Weesp.

Solvay has recently filed a DMF for CD-ISIV in the United States and intends to file an Investigational New Drug application in order to pursue clinical development. In the past two years, Solvay has consulted with the FDA on the characterization and use of the MDCK cell line for the production of influenza vaccine.

3.0 SELECTION OF THE MDCK CCL

For decades, research activities in the field of influenza and activities for surveillance of circulating influenza viruses have employed cell culture systems. These cell cultures range from primary cells to continuous cell lines, such as MDCK and Vero, which are used most often. In contrast, the influenza vaccine industry has always employed embryonated chicken eggs as a substrate for influenza virus propagation, because of the lack of technologies to employ cell culture systems at a larger scale and the absence of commercial incentive to develop new influenza vaccines. Rise in vaccine demand has been addressed by increasing the quantity of embryonated eggs processed.

The inherent limitations of the egg-based production of vaccines have motivated a search for more flexible vaccine production technologies over the past decade. New technologies include the use of mammalian continuous cell culture systems for two main reasons: (1) the economy of scale of cell culture and (2) the option of stocking all materials at the outset, which greatly improves manufacturing logistics and eliminates the need for long-term planning for the supply of eggs.

Historically, the use of cell-based biological products has raised quality concerns based on the presence of adventitious contaminants or specific properties of the cells used for production. These concerns led the U.S. Armed Forces Epidemiology Board in 1954 to recommend using "normal" cells, rather than human tumor–derived cell lines, for adenovirus vaccine development, mainly because of concerns about transmission of oncogenic agents. The recommendation created a precedent that still affects regulatory assessment of the use of continuous cell lines for vaccine development [iv]. However, knowledge of the use of and risks associated with continuous cell lines as vaccine cell substrate has increased significantly over the last 50 years.

In 1995, the WHO informal consultation on "Cell Culture as a Substrate for the Production of Influenza Vaccines" concluded that on the basis of the evidence available, MDCK and Vero were suitable for further consideration.

Solvay chose MDCK over Vero because of higher yields. MDCK cultures produce 5 times as much influenza virus as comparable Vero cultures, in half the time [v]. This translates to 10 times the production capacity.

The MDCK cell line was derived from an apparently healthy adult female cocker spaniel and established as a cell line by Madin and Darby in 1958 [vi,vii]. The source of the transformation to CCL is unknown, in contrast to establishment of more recent CCLs by genetic engineering. The potential risks associated with oncogenes in the MDCK cell DNA are, however, efficiently eliminated in downstream processing through DNA removal and DNase treatment. The canine origin as such is not a source of concern with regard to extraneous contaminants, such as HIV or BSE-like agents. Cell lines from humans or other primates pose a higher risk of containing yet-unidentified extraneous agents of potential danger to humans.

The MDCK cell line was used primarily for virological studies in the early 1960s, followed by characterization studies of the cell line itself as early as 1966 [viii]. It is one of the most-studied polarized cell lines and already was considered one of the best characterized epithelial cell lines in 1979, still closely resembling kidney epithelia in terms of morphology and growth regulation after almost 20 years of culture *in vitro* [ix, x]. MDCK cells appear to have favorable influenza virus propagation characteristics and have been used extensively for influenza research and surveillance.

Solvay Pharmaceuticals obtained MDCK cells from the ATCC at passage 52 in 1992 and developed serum-free culture conditions. These MDCK cells were banked by BioReliance Biotech (Rockville, MD) and validated both at master cell bank (MCB) passage 56 and working cell bank (WCB) passages 57 and 58 and at passage 97 for the extended cell bank (ECB). Testing was conducted in accordance with U.S. and EU regulatory guidelines. Solvay Pharmaceuticals traced the cell line history, including source and origin of employed materials, back to 1964. This track record shows that all bovine materials from 1964 onwards were from certified sources, diminishing the risk of introduction of BSE agents.

Although MDCK had been used extensively for influenza virus propagation, the production process had not previously been scaled for use of larger bioreactors in industrial applications. Solvay Pharmaceuticals developed innovative ways to overcome this challenge and successfully developed a cell culture–based influenza vaccine manufacturing process, employing MDCK cell culture in industrial-scale bioreactors under serum-free conditions. The serum-free media used for manufacture of CD-ISIV do contain a few bovine-derived components, all of which are from certified sources in Australia, New Zealand, or the United States and comply with the current guidelines for transmissible spongiform encephalopathy (TSE). Solvay Pharmaceuticals currently develops media without any animal-derived components, to exclude any risk of introduction of extraneous agents. Typical bioreactor cell cultures show yields up to 8 million cells per milliliter and growth curves comparable to those found in small-scale bioreactors[v]. All WHO-recommended vaccine viruses from the period 1992 to 2005, in many cases both the wild type and high-growth reassortant, have been successfully grown on MDCK cells.

3.1 Master Cell Bank Generation

The cell line MDCK (NBL-2) CCL-34, passage 52, batch F-9261, was received frozen from the ATCC and stored in vapor-phase nitrogen at all times except when being used. Before propagation, cell lysate samples tested negative for bacterial and fungal contamination and for agar cultivable and noncultivable mycoplasmas.

The ATCC cell line was subcultured in four passages to produce an MCB at passage 56 (MCS-MDCK ATCC-52 DU 4 10 4/92) by Microbiological Associates (now BioReliance Biotech), Rockville, MD. The cells were propagated via standard culture procedures, and a serum-free medium, EpiSerf, was employed to circumvent inter-batch variation and the introduction of adventitious viruses originating in fetal calf serum. The cell suspension was stored in glass ampules containing 1 ml at -70°C ± 10°C, sent to Solvay Pharmaceuticals, Weesp, and stored in vapor-phase nitrogen.

3.2 Working Cell Bank Generation

The MCB was subcultured in one passage to produce a WCB at passage 57 (WCS-MDCK ATCC-52 DU 5) by Microbiological Associates, Rockville MD. At the time the MCB was frozen, part of the culture was taken to produce the WCB. The cells were propagated via standard culture procedures in EpiSerf medium. The cell suspension was stored in glass ampules containing 1 or 5 ml at -70°C ± 10°C, sent to Solvay Pharmaceuticals, Weesp, and stored in vapor-phase nitrogen.

3.3 Extended Cell Bank Generation

To investigate the perpetuation of the integrity of the cell line, a post-production cell bank, or ECB, was produced and analyzed. The WCB was subcultured in 40 passages in a stirred bioreactor with microcarriers as support, to produce the ECB at passage 97. The ECB was propagated by the Solvay Research Centre at Neder-over-Heembeek, Brussels, Belgium. EpiSerf medium was used, and the applied culture conditions were representative of those at the scale intended for commercial production.

4.0 CELL BANK CHARACTERIZATION

Characterization of CCLs consists of testing for the absence of adventitious agents, characterization of the MCB and ECB, and determination of *in vivo* tumorigenic potential.

4.1 Adventitious Agent Testing

The tests used to detect the presence of adventitious contaminants form part of the characterization recommended by the FDA Center for Biologics Evaluation and Research (CBER) (Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals, 1987) and EU guidance.

The ECB was subcultured further to provide sufficient material for the various analytical procedures.

The MDCK cell line was obtained from ATCC and is a well-established cell line. During cell expansion to generate the MCB, WCB, or ECB (4 to 40 passages), no evidence of poor growth or any other cytopathic effect resulting from a viral infection was observed. Because the cell expansion was pursued over a long period (> 100 days), it is highly probable that any canine virus endogenously present in the cell line or any other virus introduced during Good Manufacturing Practice cell banking would have resulted in growth retardation, hemagglutination, and/or cytopathic effects. It is considered unlikely that canine viruses are present in the ATCC cell line or that they were introduced during the cell banking.

The MCB and ECB, produced as the result of 40 passages of the WCB, were examined for the presence of adventitious contaminants. Both cell banks were examined for the presence of mycoplasmas, bacteria, fungi, and adventitious viruses, via the test for bacterial and fungal contaminants, sterility test, and *in vivo* and *in vitro* assays for the presence of inapparent viruses.

Furthermore, the *in vitro* and *in vivo* assays for adventitious viruses were assessed for their suitability for detecting canine, bovine, and porcine viruses that might be present in the cell bank system as contaminants originating from the original cell line seed or from contaminated animal sera, culture media, or trypsin employed during subculturing of the original cell seed. This assessment identified several viruses that were not likely to be detected by the applied nonspecific assays; therefore, these were included in a canine antibody production test, in which dogs of six different breeds were inoculated with lysate of MDCK cells.

The following tests for adventitious agents were conducted with the MCB and ECB:

Microbial sterility
Mycoplasmas:
- Agar cultivable and noncultivable
- Aerobic and non-aerobic

Bacteria and fungi:
- Growth on various broths

Extraneous and endogenous agents
Endogenous and adventitious viruses:
- *In vitro:* co-cultivation in the following cell lines:
  - MRC-5 (human lung), HeLa (human carcinoma), Vero (monkey kidney), RK-13 (rabbit kidney), MDBK (bovine kidney) and MDCK (canine kidney, test cell line)
- *In vivo:* suckling mice, adult mice, guinea pigs, rabbits, and embryonated eggs (allantoic, chorioallantoic, and yolk-sac inoculation routes)
- Canine antibody production test (in dogs of six breeds)

Retroviruses:
- Induction reverse transcriptase
- Electron microscopy
- Product-enhanced reverse transcriptase (tested only for the ECB)

The results of all tests were negative. No evidence was found for the presence of microbial or viral contaminants in either the MCB or the ECB.

4.2 Genetic Integrity of Cells

The MCB and ECB were characterized and evaluated separately for genetic integrity. To confirm the identity of the cell line, the MCB and ECB were characterized by isoenzyme and cytogenetic analysis. Based on isoenzyme migration, ploidy distribution and karyology, both MCB and ECB were concluded to be of canine origin. No evidence for presence of another species was found.

Chromosome count/ploidy distribution and structural chromosome aberrations were also analyzed. The examination did not reveal any chromosomal aberrations in either the MCB or ECB. None of the chromatids or chromosomes had been interchanged or deleted and no cells had been severely damaged. The modal chromosome number reported for the original ATCC cell line is 78, the results for the MCB are 77 and 80, and for the ECB 70.

In summary, the results of isoenzyme analysis and genetic analysis were consistent with the canine origin of the MCB and ECB.

4.3 Evaluation of Tumorigenic and Oncogenic Potential of Intact MDCK Cells and Cellular Components

The tumorigenicity of intact MDCK cells was tested in athymic nude mice at a dose level of 10^7 cells in 1993 as part of the package of testing of the established cell bank. Solvay Pharmaceuticals' first consultation with CBER on the use of a CCL as substrate for influenza vaccine production took place in September 1998. CBER recommended further evaluation of the tumorigenic potential of cellular components (homogenized fractions). The study designs for testing of homogenized cells and purified DNA in nude mice, rats, and hamsters and interim results were discussed with CBER at various points in 2003–04, and the program was set up in accordance with CBER's "Defined-Risks Approach to the Assessment of Neoplastic Cells as Substrates for Viral Vaccine Manufacture"[iv] and adapted with CBER's recommendations. This approach is based on the following steps:

1. Determination of the quantity of cells needed to induce nodule development in immune-compromised test animals (athymic nude mice) and characterization of nodules (progressive or nonprogressive, invasive or noninvasive, metastases or no metastases, CCL or recipient cells).
2. Evaluation of the tumorigenic potential of MDCK cellular components and DNA in similar test systems as for the intact cells but also in test animals with a normal but immature immune system (newborn rats and newborn hamsters).
3. Quantification of the clearance of intact cells, cellular components, and cellular DNA by relevant steps of the vaccine production process.
4. Calculation of the likelihood of tumor development in a vaccine recipient from steps 1 through 3.

In testing with intact MDCK cells, nodule development (neoplastic growth) was detectable only in athymic nude mice after subcutaneous (s.c.) inoculation with $\geq 10^5$ cells. Exposure of suckling mice, adult mice, guinea pigs, rabbits, and dogs to large quantities of intact cells ($\geq 10^7$) did not lead to neoplastic growth at the injection site or to metastases (see Table 4.1).

In studies to characterize the tumorigenic potential of homogenized cellular components administered s.c., exposure of athymic nude mice, newborn hamsters, and newborn rats to homogenized MDCK cells did not lead to any tumor development (see Table 4.2).

In studies to characterize the oncogenic potential of purified MDCK cell DNA administered s.c., exposure of athymic nude mice, newborn hamsters, and newborn rats did not lead to any tumor development that could be attributed to the test material. Murine retrovirus–associated lymphomas were observed at necropsy in two of 48 nude mice six months after inoculation with high levels of MDCK DNA; these lymphomas are not considered to be related to MDCK DNA exposure, as they are not abnormal for this animal at this age. Similar background occurrences were reported by others when testing the phenotype of PER.C6 cells (Vaccine Cell Substrate 2004 meeting, Rockville, MD, 2004 [xi]). It should be noted that the validity and conclusiveness of this type of study remains the subject of scientific debate,

especially because positive control material to validate the model is not available (see Table 4.3).

Testing of MDCK cells and cellular components for tumorigenic and oncogenic potential is summarized in Tables 4.1 (intact cells), 4.2 (homogenized cells), and 4.3 (MDCK DNA).

Table 4.1 — Testing of Intact MDCK Cells for Tumorigenic and Oncogenic Potential

Report #	Test animals	Summary	Results
MAI 1992, ZB342.999032 MAI 1993, ZB387.999034	Suckling mice	Two groups of eight suckling mice, one day old, were each inoculated with 0.1 ml of test article intraperitoneally (i.p.), 0.01 ml orally, and 0.01 ml intracranially. The lactating females were untreated. An equal number of negative controls were used. After 14 days' observation, homogenates of the suckling mice were prepared, after the removal of the skin and gastrointestinal tract, and pooled per group for passage to a group of at least six two-day-old mice. The 20% w/v homogenates were administered in the same quantities and by the same routes as above. The mice were observed for 14 days.	No tumor development was observed in suckling mice exposed to intact cells or in mice exposed to the homogenates.
	Mice (Adult)	Ten adult mice, five of each sex, were each inoculated with 0.5 ml of the test article i.p., 0.05 ml orally and intranasally, and 0.03 ml intracranially. An equal number of negative controls of each sex was used. The mice were observed for 28 days.	All mice appeared to be normal and healthy during the 28-day observation period.
	Guinea pigs (Adult)	Six guinea pigs, three of each sex, were each inoculated with 5.0 ml of test article i.p. and 0.1 ml intracranially. Two negative controls of each sex were used. The animals were observed weekly for 28 days.	All guinea pigs appeared normal and healthy during the 28-day observation period.
V98.931	Rabbits (Adult)	Five young adult male rabbits were inoculated intramuscularly (i.m.) with 10^7 cells. The animals were observed weekly for 28 days.	All rabbits appeared normal and healthy during the 28-day observation period.

Table 4.1 — Testing of Intact MDCK Cells for Tumorigenic and Oncogenic Potential

H.201.04.0002	Dogs (10-12 weeks old)	Six healthy non-vaccinated SPF beagle dogs were inoculated i.m. with 10^8 cells. Two similar dogs were used as controls. At study day 42, all dogs were sacrificed, and complete necropsies were conducted.	No macroscopic abnormalities were found in any of the dogs.
MAI 1993, ZB387.999034	Nude mice (4 weeks old)	Groups of 10 athymic nude mice were inoculated with 0.2 ml of 5 x 10^7 MDCK cells/ml (at passage level 45 from MCB), Syrian hamster embryo cells at passage 39 (negative control), or 18C1-T0T cells at passage 6 (positive control). The animals were observed and the inoculation site was palpated and examined for lesions twice a week for 28 days.	The 10 mice inoculated with MDCK cells had palpable inoculation-site lesions by day 28. The masses inoculation-site masses were described as cystadenocarcinoma-type neoplasias.
H0201.7.002X	Nude mice (4 weeks old)	Groups of 13 to 20 male and 13 to 20 female mice were inoculated s.c. with 10^1,10^3, 10^5,or 10^7 MDCK cells. Additional groups of 13 mice of each sex were inoculated with 10^7 cells from the reference ATCC MDCK cell line or with 10^7 HeLa cells (positive control). The mice were observed for about six months.	No evidence of nodule or tumor formation was found at the inoculation site, lymph nodes, spleen, kidneys, brain, lungs, or liver in mice inoculated with 10^1 or 10^3 cells. Nodule formation was observed in 9 of 13 female and 9 of 13 male mice inoculated with 10^5 cells and in all 20 female and male mice inoculated with 10^7 MDCK cells. Nodule formation was observed in 23/26 mice inoculated with ATCC MDCK cells and 25/26 mice inoculate with HeLa cells.

Table 4.2 — Testing of Homogenized MDCK Cells for Tumorigenic and Oncogenic Potential

Report #	Test animals	Summary	Results
H0201.7.032X	Nude mice (4 week old)	Groups of 12 to 14 male and 12 to 14 female nude mice were inoculated s.c. Culture medium and inactivated virus concentrate were used as separate negative controls. The mice were observed for about six months.	No evidence of nodule of tumor development was found.
H0201.7.033X	Hamster (3-6 days old)	Groups of 17 to 20 male and 12 to 14 female hamsters were inoculated s.c. Culture medium and inactivated virus concentrate were used as separate negative controls. The hamsters were observed for about six months.	No evidence of nodule of tumor development was found.
H0201.7.034X	Rats (4-7 days old)	Groups of 14 to 18 male and 12 to 14 female rats were inoculated s.c. Culture medium and inactivated virus concentrate were used as separate negative controls. The rats were observed for about six months.	No evidence of nodule of tumor development was found.
H0201.7.040X	Nude mice (0-4 days old)	Groups of 14 female and 16 male nude mice were inoculated s.c. Culture medium was used as the negative control. The mice were observed for about six months.	No evidence of nodule of tumor development was found.
H0201.7.038X	Hamster (0-4 days old)	Groups of 13 female and 13 male hamsters were inoculated s.c. Culture medium was used as the negative control. The hamsters were observed for about six months.	No evidence of nodule of tumor development was found.
H0201.7.039X	Rats (0-4 days old)	Groups of 13 female and 13 male rats were inoculated s.c. Culture medium was used as the negative control. The rats were observed for about six months.	No evidence of nodule of tumor development was found.

Table 4.3 — Testing of MDCK Cell DNA for Tumorigenic and Oncogenic Potential

Report #	Test animals	Summary	Results
H0201.7.029X	Nude mice (4 weeks old)	Groups of 15 female and 15 male nude mice were inoculated s.c. Culture medium and murine DNA were used as separate negative controls. The mice were observed for about six months.	Two females had apparently non-test-article-related tumors in internal organs.
H0201.7.030X	Hamster (4-7 days old)	Groups of 20 female and 19 male hamsters were inoculated s.c. Culture medium and murine DNA were used as separate negative controls. The hamsters were observed for about six months.	No evidence of nodule of tumor development was found.
H0201.7.031X	Rats (4-7 days old)	Groups of 18 female and 18 male rats were inoculated s.c. Culture medium and murine DNA were used as separate negative controls. The rats were observed for about six months.	No evidence of nodule of tumor development was found.
H0201.7.037X	Nude mice (0-4 days old)	Groups of 16 female and 16 male nude mice were inoculated s.c. Culture medium was used as the negative control. The mice were observed for about six months.	No evidence of nodule of tumor development was found.
H0201.7/035X	Hamster (0-4 days old)	Groups of 15 female and 15 male hamsters were inoculated s.c. Culture medium was used as the negative control.The hamsters were observed for about six months.	No evidence of nodule of tumor development was found.
H0201.7.036X	Rats (0-4 days old)	Groups of 14 female and 14 male rats were inoculated s.c. Culture medium was used as the negative control.The rats were observed for about six months.	No evidence of nodule of tumor development was found.

5.0 CLEARANCE OF HOST CELL COMPONENTS AND VIRUSES

CD-ISIV is an inactivated subunit influenza vaccine derived from influenza virus vaccine strains propagated on MDCK cells. These cells are derived from the parent MDCK cell line and have retained their anchorage-dependent characteristics. They are cultivated under sterile conditions through the use of microcarriers as supporting surfaces for large stirred cultures. The applied cell culture medium, EpiSerf, is free of serum, to decrease the risk of introducing extraneous agents originating in animal sera. All animal-derived components employed during production of CD-ISIV come from certified sources. All bovine components come from certified sources in the United States or New Zealand and are certified by the European Directorate for Quality of Medicines to diminish risks of introduction of TSE agents. Figure 5.1 is a simplified diagram of the cell-based bulk vaccine production process.

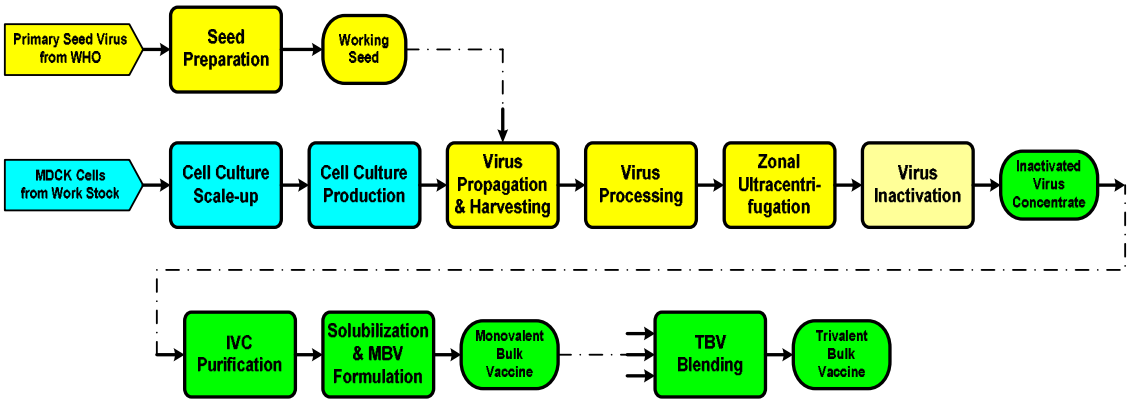

Figure 5.1 — Overall process flow diagram of CD-ISIV production

Cells are cultured to the desired volume for vaccine production and infected with the egg-adapted influenza virus vaccine strains annually recommended by WHO. The virus is allowed to propagate, harvested, purified and concentrated by clarification centrifugation, ultrafiltration, and density gradient ultracentrifugation. The purified virus concentrate is subsequently inactivated by treatment with formaldehyde.

The inactivated virus concentrate is treated with DNase to digest residual host cell DNA and further purified by chromatography and ultrafiltration. The purified virus is solubilized by treatment with detergents and ultracentrifuged to remove the viral core; the viral membrane antigens hemagglutinin and neuramidinase are recovered in the supernatant. Detergents are removed, followed by formulation and sterilizing 0.2-µm filtration to produce a monovalent bulk vaccine.

The above process is repeated for each of the vaccine strains recommended for the particular season (usually three), and the resulting monovalent bulk vaccines are aseptically blended in the correct amounts to produce the final multivalent (usually trivalent) bulk vaccine with a final potency of at least 15 µg of hemagglutinin per strain per 0.5-ml dose. The final bulk vaccine is aseptically filled in syringes in isolator filling lines.

5.1 Removal of Intact, Viable Cells

The CD-ISIV manufacturing process contains specific steps that effectively remove intact cells, i.e., homogenization, clarification centrifugation, ultracentrifugation, and three final sterilizing filtrations. Some of these steps were designed specifically for the CD-ISIV purification process, in addition to the steps used in egg-based vaccine purification. In addition, virus infection, formaldehyde treatment, and solubilization with detergent effectively kill viable MDCK cells. Clearance of intact cells has been demonstrated on a development scale and will be validated on the scale intended for commercial production.

5.2 Removal of Residual DNA

The CD-ISIV manufacturing process contains specific steps that effectively remove or digest DNA that is not cleared together with intact cells, i.e., a dual DNAse treatment and solubilization/ultracentrifugation. Some of these steps were designed specifically for the CD-ISIV purification process. Residual DNA levels on the pilot scale typically are below 100 pg per dose, well below the recommended level of 10 ng per dose. In addition, residual DNA is well fragmented by DNAse treatment. Both DNA clearance and fragmentation of residual DNA will be validated on the scale intended for commercial production.

5.3 Removal of Extraneous Agents

The major virus clearing procedure during the CD-ISIV purification process is virus inactivation by formaldehyde treatment. Also, the detergent treatment to solubilize the virus particles has the capacity to inactivate enveloped viruses. Non-enveloped viruses are effectively removed by chromatography and ultracentrifugation. Some of these steps were designed specifically for the CD-ISIV purification process.

The model viruses applied in viral clearance validation studies of the CD-ISIV purification process were herpes simplex virus type 1 (or pseudorabies virus as an alternative from the same virus family), influenza, poliovirus, and reovirus. This panel represents a broad range of viruses in terms of genome type, size, and morphology. Clearance of mycoplasmas also was validated. The CD-ISIV purification process safeguards effective and redundant clearance of all model viruses and mycoplasmas.

5.4 Potential Viral Contamination Introduced with the Influenza Seed Virus

Seed viruses for CD-ISIV production originate from the annually recommended vaccine reference viruses, which are distributed by WHO reference centers after passaging through chicken eggs and currently are wild type or egg-based high-growth reassortant strains. These vaccine reference viruses are used for all inactivated influenza vaccines worldwide. Thus, all influenza seed viruses destined for propagation by Solvay Pharmaceuticals will previously have been passaged on eggs. The initial collection of influenza viruses in the field inherently involves the co-isolation of any viruses present with the influenza virus in the upper respiratory tract. The types of viruses potentially co-isolated therefore are expected to be respiratory-tract pathogens, systemic diseases spread by respiratory dissemination, or viruses present in saliva of subjects in a persistent carrier state. The human viruses that meet these criteria for potential co-isolation with the influenza field strain were assessed for their ability to replicate in eggs and MDCK cells, based on the information found concerning the viruses' host ranges, tissue tropisms, and requirements for specific cell surface receptors. The viruses of primary concern are considered to be those that can replicate in both eggs and MDCK cells; the influenza seed viruses are tested for presence of each of these viruses by virus-specific polymerase chain reaction.

A co-isolated virus remains present as an influenza seed virus contaminant only when it is capable of being replicated in eggs, as all non-replicating viruses are cleared via dilution during egg passages. Depending upon the physical-chemical stability of the particular virus, some additional viral inactivation can be expected as a result of exposure to repeated

incubation conditions. During egg passages from field strain to primary seed virus, dilution factors typically exceed 10^{25}. This will effectively clear all contaminating viruses that do not replicate in eggs, because titers of contaminating viruses co-isolated with the influenza field strain are not likely to exceed 10^8 plaque-forming units per milliliter.

6.0 RISK ASSESSMENT

Solvay performed an assessment to identify risks associated with adventitious agents, intact viable cells, cellular components, and cellular DNA in accordance with FDA's publication "A Defined-Risks Approach to the Regulatory Assessment of the Use of Neoplastic Cells as Substrates for Viral Vaccine Manufacture." [iv] This comprehensive assessment detected no significant risks with the use of the MDCK cell line.

6.1 Risk Assessment for Adventitious Agents

As described in Section 4.1, the MDCK cell bank system was assessed for the absence of adventitious agents in accordance with European and U.S. guidelines. The assessment included the following tests:

Microbial sterility

Mycoplasmas:
- Agar cultivable and noncultivable
- Aerobic and non-aerobic

Bacteria and fungi:
- Growth on various broths

Extraneous and endogenous agents

Endogenous and adventitious viruses:
- *In vitro:* co-cultivation in the following cell lines:
  - MRC-5 (human lung), HeLa (human carcinoma), Vero (monkey kidney), RK-13 (rabbit kidney), MDBK (bovine kidney) and MDCK (canine kidney, test cell line)
- *In vivo:* suckling mice, adult mice, guinea pigs, rabbits, and embryonated eggs (allantoic, chorioallantoic, and yolk-sac inoculation routes)
- Canine antibody production test (in dogs of six breeds)

Retroviruses:
- Induction reverse transcriptase
- Electron microscopy
- Product-enhanced reverse transcriptase (tested only for the ECB)

The results of all tests were negative. No evidence was found for the presence of any adventitious agent in the MDCK cell banks.

As discussed in Section 4.2, the MCB and ECB were characterized and evaluated separately for genetic integrity. The results of isoenzyme analysis and genetic analysis were consistent with the canine origin of the MDCK cell banks.

As discussed in Section 4.3, the oncogenicity of cellular components of MDCK and of tumorviruses or tumorvirus sequences potentially present in the cell substrate was evaluated by inoculating 4-week-old and newborn athymic nude mice, newborn rats, and newborn hamsters subcutaneously with a lysate of 10^7 viable cells at a passage level higher than the maximum intended for production. Nodule development was not observed in any of these groups up to six months. Cellular components of MDCK evidently do not lead to transforming events even in immunodeficient animals, and no evidence was found for the presence of oncogenic viruses.

These studies demonstrate that the MDCK cell bank is free of adventitious agents. In addition, a viral clearance validation has demonstrated the capacity of the purification process to remove or inactivate any type of virus, thus further reducing the potential risk associated with adventitious agents to recipients of CD-ISIV. This validation used model viruses representing a range of size, morphology, and genome types, including enveloped and non-enveloped viruses. It can be concluded that the risk of exposure to an adventitious virus in CD-ISIV is negligible, because of a double safeguard: the vaccine cell substrate was shown to be devoid of virus, and the purification process has redundant capacity to clear any virus potentially present.

6.2 Risk assessment for Intact, Viable Cells

We previously reported that s.c. inoculation of immunodeficient mice with 10^7 viable cells of the MDCK cell line led to nodule formation at the injection site only [xii]. As discussed in Section 4.3, the tumorigenic potential of the cell line has been further quantified by s.c. inoculation of 4-week-old athymic nude mice with a dilution series of 10^7, 10^5, 10^3, and 10^1 viable MDCK cells derived from the ECB. The results are summarized in Table 6.1.

Table 6.1 — Nodule Development in 6-Month Tumorigenicity Studies with Intact MDCK Cells

	Animals with nodules at site of injection/total animals	Day of first observation of nodule development
MDCK, 10^1	0/26	none
MDCK, 10^3	0/26	none
MDCK, 10^5	18/26*	22
MDCK, 10^7	40/40*	5
MDCK-ATCC, 10^7	23/26*	5
HeLa, 10^7	25/26+	5

* Animals were sacrificed after six months. Nodule regression was observed in all MDCK groups.
+ Positive control. Animals were sacrificed by day 76 because of aggressive growth of tumors.

The theoretical risk of using such neoplastic cells for vaccine production has been extensively discussed in the literature [iv, xi, xiii] and in CBER Vaccines and Related Biological Product Advisory Committee meetings [xiv, xv]. The risk can be further assessed through evaluation of the chance of exposure of a vaccine recipient to a tumorigenic dose of the vaccine cell substrate. This risk is assessed by validation of the capacity of the influenza subunit vaccine purification process to clear intact cells and thus the tumorigenic potential.

Validation on the pilot scale has shown high redundancy in the capacity to clear intact cells by clarification, chemical inactivation, solubilization, ultracentrifugation, and final sterile filtration procedures. This redundant cell substrate clearance capacity will be confirmed on the intended commercial scale.

The capacity of the vaccine purification process to clear the tumorigenic potential observed with intact cells was further demonstrated by administration of early in-process materials generated during vaccine purification to a control group in the lysate oncogenicity studies in 4-week-old athymic nude mice, newborn rats, and newborn hamsters. None of the early in-process materials induced nodule development in any of the species in six months, demonstrating that the earliest purification procedure — clarification centrifugation of a crude, homogenized harvest of 10^7 cells — is sufficient to remove the tumorigenic potential of the vaccine cell substrate. Subsequent stringent purification procedures ensure sufficient redundancy and completely eliminate the risk of exposure of a vaccine recipient to the cell substrate's tumorigenic potential.

6.3 Risk Assessment for Cellular DNA

In order to show that nodule development observed in immunodeficient mice inoculated with viable MDCK cells was not caused by a transforming event originating from oncogenic genomic sequences, the oncogenicity of MDCK DNA was assessed as well, as described in Section 4.3. Four-week-old and newborn athymic nude mice, newborn rats, and newborn hamsters were inoculated with at least 100 µg of high-molecular-weight DNA extracted from cells derived from the ECB. Animals were observed for six months, and nodule development was not observed.

Clearance of DNA by the CD-ISIV purification process has been validated; levels of residual MDCK DNA typically were reduced to below the recommended level of 10 ng per dose. In addition, residual DNA is well fragmented by DNase treatment. Thus, in the studies discussed above, exposure of susceptible animals to a quantity of MDCK DNA equivalent to that in at least 10,000 vaccine doses did not reveal any oncogenic potential.

7.0 CONCLUSION

Continuous cell lines such as MDCK are desirable alternatives to embryonated chicken eggs as substrates for influenza vaccine production. In contrast to the use of embryonated eggs, cell-based manufacturing allows for a better-controlled substrate and a closed production process utilizing bioreactors. Egg-based influenza vaccine production is dependent on the availability of embryonated eggs, which is at risk in the event of outbreaks of bird diseases. In addition, a cell line could allow manufacturers to respond more quickly to the emergence of new strains of influenza in a pandemic situation. Establishment of cell-based influenza vaccine production methods is supported by the NIH, WHO, and CDC.

The characterization of Solvay Pharmaceuticals' MDCK CCL as a cell substrate for the production of influenza vaccine has involved comprehensive testing for adventitious agents and assessment of the risks associated with intact cells, cellular components, and cellular DNA. Based on our characterization of our MDCK cell line and the purification procedures

during downstream processing, we conclude that the use of Solvay Pharmaceuticals' MDCK continuous cell line provides a safe, well-controlled, viable alternative substrate for producing purified, inactivated subunit influenza vaccine.

8.0 REFERENCES

[i] Meltzer MI, Cox NJ, Fukuda K. The economic impact of pandemic influenza in the United States: priorities for intervention. Emerg Infect Dis 1999; Sep-Oct 5: 659-71.

[ii] Advisory Committee on Immunization Practices (ACIP). Recommendations: Prevention and Control of Influenza. MMWR April 25, 2003; 52(RR08): 1-36.

[iii] Advisory Committee on Immunization Practices (ACIP). Recommendations: Prevention and Control of Influenza. MMWR April 25, 2003; 52(RR08): 1-36.

[iv] Lewis AM Jr, Krause P, Peden K: A defined-risk approach to the regulatory assessment of the use of neoplastic cells as substrates for viral vaccine manufacture, in Brown F, Lewis AM, Peden K, Krause P (eds): Evolving Scientific and Regulatory Perspectives on Cell Substrates for Vaccine Development. Dev Biol. Basel, Karger, 2001, 106: 513-535.

[v] Merten OW, Hannoun C, Manuguerra JC, Ventre F, Petres S. Production of influenza virus in cell cultures for vaccine preparation. In Cohen S, Schafferman A (eds.): Novel strategies in design and production of vaccines. Plenum Press, New York, 1996; 141-151.

[vi] Madin SH, Darby NB. Proc Soc Exp Biol Med 1958; 98: 574-576.

[vii] U.S. Dept. HEW, P.H.S. Registry of Animal Cell Lines, 1st Ed., 1st Suppl. 1965.

[viii] Gaush CR, Hard WL, Smith TF: Characterization of an established line of canine kidney cells (MDCK), Proc Soc Exp.Biol Med 1966; 122: 931-935.

[ix] Taub M, Saier, MH Jr: An established but differentiated kidney epithelial cell line (MDCK), Methods Enzymol 1979; 58: 552-560.

[x] Taub M, Ue B, Chuman L, Rindler MJ, Saier MH Jr: Alterations in growth requirements of kidney epithelial cells in defined medium associated with malignant transformation, J Supramolec. Struc. 1981; 15: 63-72.

[xi] Petricciani J, Sheets R (eds): Vaccine Cell Substrates 2004. Dev Biol. Basel, Karger, 2005, vol 123 – pending for publication.

[xii] Brands R, Visser J, Medema J, Palache AM, van Scharrenburg GJM: Influvac^{TC}: A Safe Madin Darby Canine Kidney (MDCK) Cell Culture-Based Influenza Vaccine, in Brown F, Robertson JS, Schild GC, Wood JM (eds): Inactivated Influenza Vaccines Prepared in Cell Culture. Dev Biol Stand. Basel, Karger, 1999, vol 98: 93-100.

[xiii] Brown F, Lewis AM, Peden K, Krause P (eds): Evolving Scientific and Regulatory Perspectives on Cell Substrates for Vaccine Development. Dev Biol. Basel, Karger, 2001, vol 106.

[xiv] Center for Biologics Evaluation and Research – Vaccines and Related Biological Products Advisory Committee on November 19, 1998.

[xv] Center for Biologics Evaluation and Research – Vaccines and Related Biological Products Advisory Committee on May 16, 2001.