EVALUATION OF METHODS OF AVIAN LEUCOSIS VIRUS INACTIVATION IN PRODUCTION OF INFLUENZA VACCINES

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The process of production of inactivated influenza vaccines involves a stage of inactivation of both the influenza virus and the possible viral contaminants that can come from the raw materials (chicken embryos). One of such contaminants is the avian leucosis virus. The minimum viral contaminant load reduction that the inactivating agents should guarantee is by 4 lg/ml; this or higher level of the deactivating ability ensures the finished vaccine is free from viral contaminants. The purpose of this work was to cultivate the leucosis virus to the titer of 5 lg/ml (minimum) and to measure the reduction of the avianleucosis virus titer in influenza vaccine intermediates upon exposure to the inactivating agents. The RAV-1 and RAV-2 leucosis virus strains and influenza vaccine intermediates such as virus-containing allantoic fluid and virus concentrates were used in the study. Avian leucosis virus titers were determined by enzyme immunoassay. We created conditions for cultivation of the RAV-1 and RAV-2 avian leucosis virus strains in the primary culture of chicken embryo fibroblasts (CEF); the inactivating agents considered were the most commonly used β-propiolactone and UV radiation. It was found that after 12 hours of exposure to β-propiolactone, the RAV-1 avian leucosis virus load decreased by 4.61 ± 0.46 lg, and that of RAV-2 strain — by 4.33 ± 0.33 lg, which indicates that β-propiolactone is an effective inactivating agent. Five minutes of exposure to UV radiation reduces the RAV-1 strain viral load by 4.22 ± 0.31 lg and RAV-2 strain viral load by 4.44 ± 0.48 lg.

Keywords: influenza vaccines, inactivation, avian leucosis virus, RAV-1, RAV-2, propiolactone, UV radiation

Author contribution: all authors contributed equally to the research methodology design, data collection, analysis and interpretation, article authoring and editing.

Compliance with the ethical standards: the study was conducted in accordance with the ethical principles of the Declaration of Helsinki of 1964 and its subsequent revisions.

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ОЦЕНКА МЕТОДОВ ИНАКТИВИРОВАНИЯ ВИРУСА ЛЕЙКОЗА ПТИЦ ПРИ ПРОИЗВОДСТВЕ ГРИППОЗНЫХ ВАКЦИН

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При производстве инактивированных гриппозных вакцин на стадии инактивации должен быть инактивирован как вирус гриппа, так и возможные вирусные контаминанты, которые могут попасть в вакцину из сырья (куриных эмбрионов). Одним из возможных контаминантов является вирус лейкоза птиц. Инактиваторы должны обеспечивать гарантированное снижение вирусной нагрузки контаминанта не менее чем на 4 lg/мл, что обеспечит его отсутствие в готовой вакцине. Целью работы было осуществить наработку вируса лейкоза для достижения минимального титра 5 lg/мл, оценить снижение титра вируса лейкоза птиц в полупродуктах гриппозных вакцин при воздействии инактиваторов. В исследовании использовали штаммы вируса лейкоза RAV-1 и RAV-2 и полупродукты гриппозных вакцин, такие как вируссодержащая аллантоисная жидкость и вирусные концентраты. Титры вируса лейкоза птиц определяли методом иммуноферментного анализа. Были подобраны условия наработки вируса лейкоза птиц штаммов RAV-1 и RAV-2 в первичной культуре фибробластов эмбрионов кур (ФЭК); рассмотрены основные используемые инактиваторы — β-пропиолактон и УФ-излучение. Выявлено, что спустя 12 ч инактивации β-пропиолактоном вирус лейкоза птиц штамма RAV-1 показал снижение вирусной нагрузки на 4,61 ± 0,46 lg, а вирус лейкоза птиц штамма RAV-2 — на 4,33 ± 0,33 lg, что указывает на эффективное действие β-пропиолактона при инактивации. Проведение инактивации УФ-излучением позволяет снизить вирусную нагрузку штамма RAV-1 на 4,22 ± 0,31 lg, а штамма RAV-2 на 4,44 ± 0,48 lg за 5 мин.

Ключевые слова: гриппозные вакцины, инактивация, вирус лейкоза птиц, RAV-1, RAV-2, пропиолактон, УФ-излучение

Вклад авторов: все авторы внесли равнозначный вклад в разработку методики исследования, получение, анализ и интерпретацию данных, в написание и редактирование статьи.

Соблюдение этических стандартов: исследование проведено с соблюдением этических принципов Хельсинкской декларации Всемирной медицинской ассоциации 1964 г. и последующих ее пересмотров.

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To make the end product safe, the influenza virus should be completely inactivated during production of the inactivated influenza vaccines. This is a recommendation by both the World Health Organization and the European Medical Agency [1, 2]; for vaccines of international quality, it is a requirement. Chicken embryos used in the production of influenza vaccines can potentially carry zoonotic infections, such as the avian leucosis virus, avian adenovirus, mycoplasma. The mentioned recommendation prescribes that the vaccine production technology includes measures aimed at inactivation of the listed contaminants, too.

There are various ways of inactivating viruses in production of vaccines, including those employing UV radiation, formaldehyde or β-propiolactone [3]. The efficacy of these agents against viruses differs.

An earlier research investigated the effect of inactivating agents on avian adenovirus of CELO and Fontes strains [4]: both β-propiolactone and UV radiation were found to be effective in inactivating these strains. It was established that after 10 hours of exposure to β-propiolactone, the viral load associated with the CELO strain adenovirus decreased by 4.12 ± 0.06 lg (PFU)/ml and that by the Fontes strain adenovirus — by 4.20 ± 0.19 lg (PFU)/ml, which indicates that β-propiolactone is an effective solution to the inactivation task. Five minutes of exposure to UV radiation reduces the CELO strain viral load by 4.22 ± 0.31 lg and Fontes strain viral load by 4.44 ± 0.48 lg. It was noted that detergent added at the scission stage also reduces the viral load by 0.93 ± 0.15 lg (PFU)/ml and 1.04 ± 0.12 lg (PFU)/ml in case of CELO and Fontes strains, respectively, when the substance is n-octyl-β-D-glucopyranoside, and by 1.18 ± 0.17 lg (PFU)/ml and 1.12 ± 0.38 lg (PFU)/ml when the substance is tetradecyltrimethylammonium bromide.

In this connection, it became necessary to study in detail the effect of the above agents on another possible contaminant, the avian leucosis virus. The avian leucosis virus belongs to RNA-containing oncornaviruses of the *Retraviridae* family; they cause leucosis and sarcomas in birds and include six antigenic subgroups A, B, C, D, E, J. The viruses of this group are found in tumor tissue, blood, parenchymal organs and in chicken eggs. A team of researchers has investigated the subject of avian leucosis virus in poultry farms of the Russian Federation and found antibodies thereto at 90% of the farms involved in the study [5]. Thus, there is a serious risk of contamination of eggs used in vaccine production with avian leucosis virus. It should also be noted that there is an urgent need to introduce regulations in the Russian Federation prescribing use of hatching eggs free from the avian leucosis virus in vaccine production.

The use of UV radiation to inactivate viruses has been investigated earlier, but there was only one strain considered, RAV-1 [6]. Formaldehyde is one of the potential inactivating agents, but it can jeopardize the stability of the finished influenza vaccine and its immunogenicity, since it is a highly reactive compound; moreover, formaldehyde is capable of chemically modifying the influenza virus hemagglutinin and affecting the antigenic determinants [7, 8]. Another chemical inactivating agent, β-propiolactone, does not have these drawbacks; it effectively inactivates the influenza virus and is hydrolyzed to 3-hydroxypropionic acid, an intermediate metabolite of human lipid metabolism [9], this hydrolysis being a positive factor for the safety of the vaccine.

The purpose of this study was to select an optimal agent to inactivate the contaminant of influenza vaccines, avian leucosis virus, its most common groups RAV-1 (subgroup A) and RAV-2 (subgroup B) in particular. We also aimed to find out the minimum duration of the inactivation stage that guarantees viral load reduction by at least 4 lg infectious units (IU)/ml [10].

METHODS

Materials

For our study, we used:

– avian leucosis virus RAV-1 (ATCC-VR-335) from the ATCC collection (USA);

– avian leucosis virus RAV-2 (ATCC-VR-1828) from the ATCC collection (USA);

– 10-day-old chicken embryos from Sinyavinskaya Poultry Farm (Russia);

– IDEXX ALV Ag Test (IDEXX Laboratoties, Inc., USA).

Virus-containing allantoic fluid

We used 9–11-day-old chicken embryos to cultivate the influenza virus. The embryos were infected with a dose of 0.2 ml, infectious activity 102.0–104.5, and an egg infective dose 50 (EID_{50}). Chicken embryos were incubated at 35 °C for 48 hours for type A influenza virus and for 72 hours in case of type B influenza virus. After incubation, the embryos were cooled and allantoic fluid containing the virus (AF) harvested.

Virus concentrates (VC)

We filtered AF through a cascade of filters with a pore diameter of 10.6 and 1 μm, then concentrated the resulting fluid and put it into the ultrafiltration unit with cutoff at 300 kDa. The concentrated virus-containing allantoic fluid was ultracentrifuged in the sucrose density gradient (60–20%). We harvested fractions of the sucrose gradient in the range of 40–25% of sucrose. The harvested fractions were mixed and stored at –20 °C until the study.

RAV-1 and RAV-2 avian leucosis virus strains cultivation

We used the primary fibroblast cell culture (CEF) prepared from 10-day-old chicken embryos to cultivate the avian leucosis virus strains RAV-1 and RAV-2. Production of the CEF culture followed prescriptions provided in the respective publication [11]; it was done in 75 cm^3 culture flasks.

After formation of the cell culture monolayer, we diluted the virus on a medium as needed (nutrient medium 199, with the addition of TPCK-trypsin to a final concentration of 2 μg/ml and BSA (fraction V) to a final concentration of 0.2%). The resulting virus culture dilution was introduced to the primary cell culture of CE fibroblasts (85–95% monolayer), preliminarily washed twice with PBS, in a volume of 2–3% of the mattress volume (necessary for complete coverage of the monolayer with the virus-containing liquid). Three ml of the virus culture were added to the fibroblast cell culture, the cultures left in contact for 1 hour at +37 °C. Then, we added the maintenance medium (6 ml) and incubated the culture in a CO_2 incubator at +37 °C. The medium was changed on the 5th day of incubation.

The samples (virus-containing culture fluid) were harvested on the 13th (RAV-1) or on the 7th (RAV-2) days. At the end of the incubation, the vials with the virus-containing liquid were frozen at –20 °C and left to thaw at room temperature; the freezing-thawing routine was repeated 2–3 times. Then the virus-containing liquid was taken from the vials, centrifuged at 1159 g for 10 minutes to pellet the cells.

Determination of the RAV-1 and RAV-2 avian leucosis virus titers in the CEF primary cell culture

To determine the titers of RAV-1 and RAV-2 viruses in the AF and VC in the context of modeling their inactivation with β-propiolactone (β-PL) and UV irradiation, we titrated the samples at different periods of inactivation on the CEF primary cell culture and then tested them for p27 in each dilution with the help of ELISA. Each sample was studied in triplicates. We prepared 10-fold dilutions of the samples (from 10^{-1} to 10^{-6}) cultured on the maintenance medium. The dilutions used in modeling the RAV-1 virus inactivation with β-PL or UV irradiation, as well as the negative control culture (AF and VC series free from contaminants) were added to the CEF cell culture, 0.5 ml per well (each dilution studied in triplicate), and

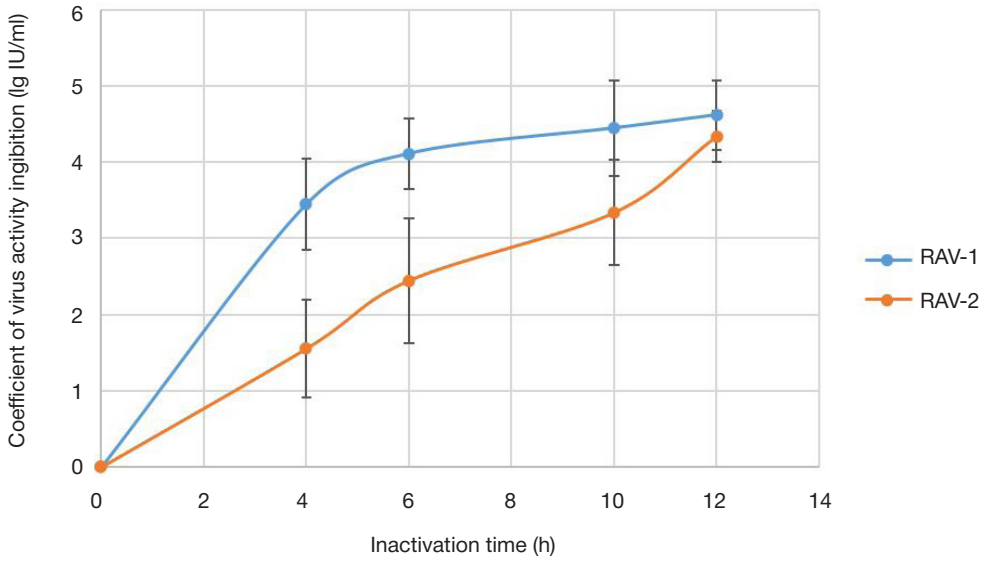

Fig. 1. Dynamics of inactivation of the RAV-1 and RAV-2 avian leucosis virus strains with β-propiolactone

left in contact for 5 hours at the temperature of +37 °C. Then the virus-containing culture fluid was removed and maintenance medium added, 1 ml per well. The plates were incubated in a CO2 incubator at +37 °C, 5% CO_2, for 7 days.

At the end of the incubation, the plates with the CEF primary cell culture were frozen at –20 °C and left to thaw at room temperature; the freezing-thawing routine was repeated 2–3 times. Then culture liquid was harvested from the wells and centrifuged at 3000 rpm to pellet the cells.

The supernatant was further tested for p27 antigen with ELISA, testing enabled by the IDEXX ALV Ag Test (IDEXX Laboratoties, Inc., USA) as per the manufacturer's instructions. We took as a titer the highest dilution of the virus-containing liquid sample that had the the optical densities ratio with the positive control greater than 0.2. The sensitivity limit of the method is 1lg IU/ml. A titer less than 1lg IU/ml was taken as 0.5.

Statistical processing of results

We processed the results with Microsoft Excel 365 (Microsoft corp.; USA) and Minitab 19 (Minitab Inc.; USA). The confidence intervals of the mean value were calculated with a confidence level of 95%.

RESULTS

Investigation of the dynamics of inactivation of avian leucosis virus in virus-containing allantoic fluid by β-propiolactone

The simulation of AF infection with the avian leucosis virus and subsequent inactivation thereof followed the pattern applied in the avian adenovirus inactivation study [4]. We added the preliminarily titrated infectious material containing avian leucosis virus to the AF samples in a volume equal to 10% of the initial sample volume, so that its final content was at least 5 lg IU/ml. The resulting contaminated samples were supplemented with β-propiolactone to a final concentration of 0.09%, and the avian leucosis virus titer in the samples was determined in accordance with the described method. Figure 1 shows the inactivation dynamics.

According to the data obtained, the viral load decreased by at least 4 lg IU/ml in at least 12 hours after supplementation with β-propiolactone at the temperature of +4 – +8 °C (Table 1).

Investigation of the dynamics of inactivation of avian leucosis virus in virus concentrates with UV radiation

The simulation of VC infection with the avian leucosis virus and subsequent inactivation thereof followed the pattern applied in the avian adenovirus inactivation study [4]. We added the preliminarily titrated infectious material containing avian leucosis virus to the VC samples in a volume equal to 10% of the initial sample volume, so that its final content was at least 5 lg IU/ml. Seven ml of the contaminated VC were placed into 90 mm Petri dishes. The dishes were then irradiated with 60 W UV light from 0, 0.5, 1, 2 and 5 minutes; the source of light was 20 cm away. The inactivation was carried out at the temperature of +18 °C.

After the specified time intervals, we removed the cups from the unit and took samples (1 ml) for further determination of the virus titer as per the described method. Figure 2 shows the inactivation dynamics.

The viral load decreased by at least 4 lg IU/ml in at least 5 minutes under UV light (Table).

DISCUSSION

According to the long-term data collected at the St. Petersburg Research Institute of Vaccines and Serums of the FMBA

Table. Reduction of the viral load by avian leucosis virus under the action of various inactivating agents

Inactivating agent	Strain	
	RAV-1	RAV-2
β-Propiolactone (inactivation time: 12 hours)	4,61 ± 0,46 lg	4,33 ± 0,33 lg
UV radiation (inactivation time: 5 minutes)	4,22 ± 0,31 lg	4,33 ± 0,48 lg

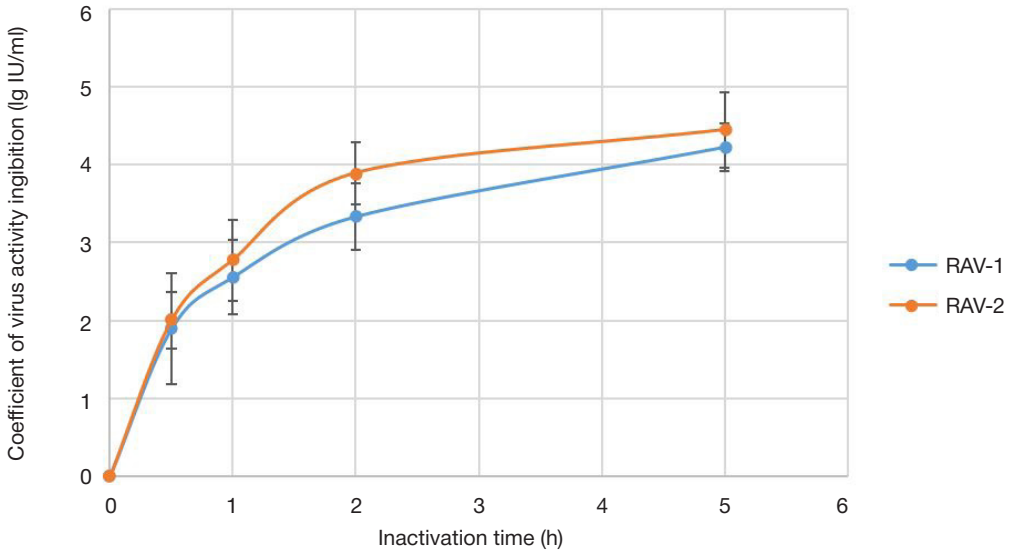

Fig. 2. Dynamics of inactivation of the RAV-1 and RAV-2 avian leucosis virus strains with UV radiation

of Russia, the term of full inactivation of the influenza virus strains that the WHO recommends for inclusion in inactivated influenza vaccines is 4–6 hours in case of β-propiolactone and 3–4 minutes under UV radiation. However, these terms do not guarantee inactivation of the possible contaminants, which makes the risk of producing unsafe vaccines real. In an earlier study [4], we have shown that both β-propiolactone and UV radiation are effective against adenovirus (strains Fontes and CELO), which is a possible a contaminant. The associated viral load decreases by more than 4 lg PFU / ml in at least 10 hours when β-propiolactone is used to inactivate AF (4.12 ± 0.06 lg and 4.20 ± 0.19 lg for CELO and Fontes, respectively) and in at least 5 minutes when the inactivating agent is UV (4.69 ± 0.89 lg and 4.44 ± 1.06 for CELO and Fontes, respectively).

According to the published research, the common inactivating agents used against avian leucosis virus are temperature and formalin (inactivation term of 24 hours) [12, 13], but they are hardly usable in production of the influenza vaccines since both temperature and formalin decrease their immunogenicity and formaldehyde itself is toxic. UV radiation has only been described as an inactivating agent for RAV-1 [6]; the reported viral load decrease was by 2 lg after 10 minutes of irradiation of virus-containing materials from a distance of 40 cm with lamps with a total power of 30 W.

This study has shown that β-propiolactone and UV radiation are also effective against RAV-1 and RAV-2 strains of the avian leucosis virus. However, the avian leucosis virus is inactivated with β-propiolactone in no less than 12 hours, while the term for adenovirus is 10 hours. Therefore, to inactivate both contaminants, the influenza vaccine production process should include the stage of inactivation with β-propiolactone that lasts at least 12 hours at a temperature of +4–+8 °C. As for the UV radiation, the lower confidence interval (p = 0.95) limit for the viral load reduction factor is less than 4 lg, which indicates the need to increase the duration of inactivation to more than 5 minutes.

Thus, inactivation with β-propiolactone ensures greater reproducibility of the results and decreases the viral load by both avian leucosis virus and avian adenovirus by more than 4 lg (guaranteed) in the process of production of influenza vaccines. Subsequently, β-propiolactone minimizes the risks and is used as an inactivating agent as part of the inactivated influenza vaccines production process adopted by various companies, e.g., by the St. Petersburg Research Institute of Vaccines and Serums of the FMBA of Russia, Novartis, GSK, ID Biomedical Corp of Quebec [14–16].

CONCLUSIONS

This study has shown that the minimum time of inactivation of the allantoic fluid containing the avian leucosis virus with β-propiolactone in the context of production of influenza vaccines is 12 hours. Through this term, the load by the avian leucosis virus grows down by 4 lg IE/ml. As for UV, the time of exposure with the aim to inactivate virus concentrates should be no less than 5 minutes, which ensures a decrease of load by the avian leucosis virus by 4 lg IE/ml. Such patterns of inactivation guarantee complete adenovirus inactivation and ensure an adequate level of safety of the produced influenza vaccines in terms of these contaminants. The next stage is to study the kinetics of mycoplasma inactivation by various inactivating agents in the process of production of influenza vaccines.

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