Using ICR and SCID mice as animal models for smallpox to assess antiviral drug efficacy

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The possibility of using immunocompetent ICR mice and immunodeficient SCID mice as model animals for smallpox to assess antiviral drug efficacy was investigated. Clinical signs of the disease did not appear following intranasal (i.n.) challenge of mice with strain Ind-3a of variola virus (VARV), even when using the highest possible dose of the virus (5.2 log_{10} p.f.u.). The 50 % infective doses (ID_{50}) of VARV, estimated by the virus presence or absence in the lungs 3 and 4 days post-infection, were 2.7 ± 0.4 log_{10} p.f.u. for ICR mice and 3.5 ± 0.7 log_{10} p.f.u. for SCID mice. After i.n. challenge of ICR and SCID mice with VARV 30 and 50 ID_{50}, respectively, steady reproduction of the virus occurred only in the respiratory tract (lungs and nose). Pathological inflammatory destructive changes were revealed in the respiratory tract and the primary target cells for VARV (macrophages and epithelial cells) in mice, similar to those in humans and cynomolgus macaques. The use of mice to assess antiviral efficacies of NIOCH-14 and ST-246 demonstrated the compliance of results with those described in scientific literature, which opens up the prospect of their use as an animal model for smallpox to develop anti-smallpox drugs intended for humans.

INTRODUCTION

According to the requirements of the Russian national control authority (Scientific Center of Expertise of Medical Application Products – SC EMAP) as well as the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), in order to assess the efficacies of developed antivirals at the stages of scientific research and clinical studies it is important to have at least two animal species simulating the corresponding infectious disease in humans. Only one animal species has been chosen for smallpox, cynomolgus macaque: M. fascicularis, synonym M. irus (Jahrling et al., 2004; Huggins et al., 2009; Chapman et al., 2010). However, this animal model is expensive and its application in virological experiments is labour-intensive, making it difficult to use in large-scale statistically significant experiments. In addition, despite the fact that the clinical picture caused by variola virus (VARV) in these primates is relatively close to that in humans, they are 1000–100 000 000 times less susceptible to this pathogen following respiratory challenge (Hahon & Wilson, 1960; Hahon & McGavran, 1961; Noble & Rich, 1969; LeDuc & Jahrling, 2001; Jahrling et al., 2004) than humans: less than 1 log_{10} p.f.u. (Drozdov et al., 1987; Zamedyanskaya et al., 2014). At the same time, these animals are used only to assess the therapeutic efficacies of anti-smallpox drugs. To do this, the animals are challenged intravenously with 8 and 9 log_{10} p.f.u. (Huggins et al., 2009; Mucker et al., 2013), whereas the main route of VARV infection in humans is via the respiratory tract. Moreover, the results of evaluating the efficacies of therapeutic and prophylactic anti-smallpox drugs using this animal model are applicable for humans with a normally functioning immune system. Some existing and emerging anti-smallpox drugs and live smallpox
vaccines (Marennikova & Shchelkunov, 2005) can be harmful for individuals with immune system pathologies, and their efficacy can differ from that in individuals without this pathology. The number of people with suppressed immune system function is growing annually due to deteriorating environmental conditions, which is associated with increasing volumes of industrial emissions, the spreading HIV epidemic, an increasing number of cancer patients requiring immunosuppressive therapy, etc.

Previously we discovered that primary lung cell cultures obtained from outbred immunocompetent ICR mice were sensitive to VARV and capable of supporting virus replication (Sergeev et al., 2013). We therefore focused on these mice in our search for an immunocompetent animal model for smallpox to study anti-smallpox drug activity. Using 10–14 day outbred ICR mice, we attempted to simulate the infectious process in infants, who are more frequently affected by smallpox than in adults (Fenner et al., 1988; Riedel, 2005a, b). In addition, outbred mice to a greater extent than inbred ones reflect the actual physiological state of the human population. Among immunodeficient animals, our attention was drawn to outbred immunodeficient SCID mice, on the one hand, having a combined immunodeficiency involving T- and B-lymphocytes and immunoglobulins (Belizário, 2009), and on the other hand, having no changes in the cells of the mononuclear phagocyte system (macrophages) and many other cells, which is extremely important for research with orthopoxviruses (including VARV) for which the main primary target cells are respiratory tract macrophages and epithelial cells.

In the current study, we investigate the possibility of using ICR and SCID mice as animal models for smallpox to assess the efficacy of anti-smallpox drugs.

RESULTS

The sensitivity of mice following intranasal challenge

In the first series of experiments, aimed at the determination of the sensitivity of ICR and SCID mice to VARV, we attempted to identify external clinical signs of the disease in animals challenged with the virus in the maximum dose of $5.2 \log_{10} p.f.u.$ Challenged mice were observed up to 21 days post-infection (p.i.) but did not display obvious clinical symptoms. To investigate the possibility of asymptomatic disease in these animals, the dynamics of the pathogen accumulation in the lungs were studied in ICR and SCID mice challenged intranasally (i.n.) with the doses of 4.2 and 5.2 $\log_{10}$ p.f.u., respectively. The results of these studies are presented in Fig. 1(a, b).

The data in Fig. 1(a, b) show that a more significant accumulation of the virus was observed 2–4 days p.i. in the lungs of ICR and SCID mice as compared with that 1 day p.i. More pronounced differences were observed in ICR mice. The 50 % infective dose (ID_{50}) of VARV was
evaluated by its presence or absence in the lungs of ICR and SCID mice 3 and 4 days p.i., respectively. It was determined that the values of this index were 2.7 ± 0.4 log10 p.f.u. for ICR mice and 3.5 ± 0.7 log10 p.f.u. for SCID mice.

**Dynamics of the virus dissemination in infected mice**

Taking into account the relatively high sensitivity of mice to VARV estimated by recording the pathogen presence in the lungs, the dynamics of VARV dissemination in different organs, tissues and serum of ICR and SCID mice following i.n. infection with the doses of 4.2 log10 p.f.u. (30 ID50) and 5.2 log10 p.f.u. (50 ID50), respectively, were studied. The results of these experiments are presented in Fig. 2(a, b), respectively.

It can be seen that VARV was detected in the lungs of ICR mice 1 day p.i. Two days p.i. it was found in high concentrations in the lungs and nasal septum with mucosa, but by day 10 p.i. the virus was not found in these tissues (Fig. 2a). At 3 and 5 days p.i. VARV was found in relatively low concentrations in the mouse brain. The virus was not detected in the cells, serum, trachea, oesophagus, liver, spleen, kidneys and duodenum at any of the time points of the study from 1 to 10 days p.i. The highest VARV concentration was recorded in the lungs and nasal septum with mucosa 2 days p.i. The highest concentrations of VARV were detected in the lungs and nasal septum with mucosa 2–4 days p.i.

The data in Fig. 2(b) show that 1 day p.i. the pathogen was detected only in the lungs of SCID mice; 2 days p.i. the pathogen was detected in relatively high concentrations

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**Fig. 2.** Accumulation dynamics of VARV strain Ind-3a in samples of (a) ICR and (b) SCID mice (n=4) challenged i.n. with the dose of 4.2 log10 p.f.u. (30 ID50) and 5.2 log10 p.f.u. (50 ID50), respectively. Virus concentration was detected for organs and tissue in 10 % (by volume) homogenates. Mean values were plotted on a log scale. *The value was determined in 10 % lung homogenate 10 min after the virus material inoculation.
in the lung and trachea and 3 days p.i. the virus appeared also in the nose (nasal septum with mucosa) while disappearing in the trachea. For 3 days (days 3–5 p.i.) VARV was detected only in the lungs and nose, and it was no longer detectable in the latter 7 days p.i., whereas in the lungs it was titrated at this time in a low concentration as compared with that of 2–5 days p.i. The virus was not detected from 1 to 7 days p.i. in other tested samples (blood cells, serum, brain, oesophagus, liver, spleen, kidneys and duodenum). The highest VARV concentrations were detected in the lungs 2–5 days p.i. when their values were within the range from 3.7 to 4.2 log10 p.f.u.

In experiments in vitro using cells of the mononuclear phagocyte system (spleen monocyte-macrophage monolayer primary culture, SMMMPC) obtained from ICR mice, infectivity assays revealed a significant increase in the biological concentration of the virus as early as 24, 48, 72 and 168 h p.i. The virus reached the maximal level of accumulation in cell cultures 48 h after challenge with the dose of 0.017 p.f.u. per cell and 168 h after challenge with the dose of 0.003 p.f.u. per cell (Fig. 3).

Pathomorphological changes in mice challenged intranasally with the virus

We further studied pathomorphological changes in ICR and SCID mice after i.n. challenge with the pathogen, using doses of 4.2 log10 p.f.u. (30 ID50) and 5.2 log10 p.f.u. (50 ID50), respectively.

At the beginning and the height of the infectious process (3–5 days p.i.), foci of vacuolar degeneration of the surface epithelium were observed in the nasal cavity of infected mice as compared to control animals (Fig. 4a, b). The number of cell layers considerably increased, and intercellular spaces seemed to be expanded. Moderate diapedesis of granulocytes and lymphocytes was sometimes observed. Noticeable structural changes in the glands, muscle elements, cartilage and bone components were not identified. Unlike control animals, examination of paraffin and ultrathin sections of trachea of infected mice during this period revealed clear signs of vacuolar degeneration of epithelium (Fig. 4c, d), generally similar to those in the nasal cavity observed in semithin sections. Moreover, changes in epithelial tissue were accompanied by pronounced mucosal oedema. All examined samples of the lungs of ICR and SCID mice 5–7 days p.i. showed pronounced inflammatory changes of the parenchyma with the predominance of intense perivascular oedema and degenerative changes in the bronchial epithelium. The pathological process equally affected both bronchial wall and alveoli. The formation of atelectasis was observed in the lungs of experimental animals, while these pathological changes were absent in control mice (Fig. 4e–h). Sometimes a pronounced cellular infiltration was accompanied by the destruction of lung tissue. Lymph nodes were randomly identified on the preparations. They showed no apparent disturbances of morphology. Unlike SCID mice, local degenerative changes in neurons of the pyramidal layer were detected only in the cortex of most ICR mice (Fig. 4i, j). The cells were in the state of balloon dystrophy and intercellular spaces were expanded to varying degrees. Inflammatory infiltration was absent. No pathological changes were recorded in other organs and tissues of ICR and SCID mice (blood cells, bifurcation...
Fig. 4. Light and electron microscopy of internal organs of ICR mice and immunodeficient SCID mice after intranasal challenge with VARV strain Ind-3a in the doses of 4.2 and 5.2 log₁₀ p.f.u., respectively, as well as SMMMPC from ICR mice 3 days p.i. with VARV in the dose 0.017 p.f.u. per cell. (a) Electronogram of nasal mucosa of ICR mouse 3 days p.i.: degenerative changes in mucosal epithelium – the loss of cilia (white arrows), vacuolization of epithelial cell cytoplasm (black arrows). (b) Electronogram of nasal mucosa of a control ICR mouse: epithelial cell cilia are not damaged (white arrows), normal epithelial cell cytoplasm (black arrows). (c) Tracheal mucosa of ICR mouse 3 days p.i.: arrows show epithelial layer with vacuolar degeneration of covering epithelial cells (histological preparation, haematoxylin and eosin staining). (d) Tracheal mucosa of a control ICR mouse: unchanged tissue (histological preparation, haematoxylin and eosin staining). (e) Lungs of ICR mouse 5 days p.i.: pronounced inflammatory with predominance of atelectasis (arrows) and intense perivascular oedema (histological preparation, haematoxylin and eosin staining). (f) Lungs of SCID mouse 5 days p.i.: diffuse oedema, vascular congestion and atelectasis in the area of the large bronchus (histological preparation, haematoxylin and eosin staining). (g) Lungs of a control SCID mouse: no changes (histological preparation, haematoxylin and eosin staining). (h) Lungs of SCID mouse 5 days p.i.: an area of atelectasis, alveolar spaces are sharply narrowed (white arrows); type II alveolocyte in the centre (black arrow) and normal lumen of the alveoli at the top (electronogram). (i) Brain of ICR mouse 5 days p.i.: local degenerative changes in neurons of the pyramidal layer; cells in the state of ballon dystrophy (arrows), intercellular spaces are expanded to varying degrees (histological preparation, haematoxylin and eosin staining). (j) Brain of ICR mouse 5 days p.i.: local degenerative changes in neurons of the pyramidal layer; cells in the state of ballon dystrophy, intercellular spaces are expanded to varying degrees (electronogram). (k) Mouse spleen monocyte–macrophage from a primary culture of macrophage-like cells prepared in vitro: numerous viral particles can be seen in the cytoplasm of the destroyed cell (electronogram). (l) Close-up of an area of monocyte–macrophage cytoplasm (see the previous micrograph) demonstrating a typical ultrastructure of mature viral particles (electronogram).
lymph nodes, liver, spleen, pancreas, duodenum, mesenteric lymph nodes, kidneys, adrenals and skin pieces) collected at different time points.

In in vitro experiments, electron microscopy revealed signs of VARV reproduction in cells of the mononuclear phagocyte system (spleen monocytes–macrophages) obtained from ICR mice (Fig. 4k, l).

Drug efficacy in experiments in mice challenged intranasally with the virus

The next series of experiments assessed the therapeutic and prophylactic efficacies of ST-246 and NIOCH-14 in ICR and SCID mice challenged i.n. with VARV, at doses of 3.7 log_{10} p.f.u. (10 ID_{50}) and 4.5 log_{10} p.f.u. (10 ID_{50}), respectively. Tables 1 and 2 present the results of these studies.

It was noted (Table 1) that 3 days p.i. the number of infected ICR mice treated with ST-246 and NIOCH-14 and challenged with VARV was significantly (P<0.05) lower than in the control group. Moreover, the drugs reduced VARV production in mouse lungs 3 days p.i. as compared with the control.

The data in Table 2 show that the number of SCID mice treated with NIOCH-14 in which VARV concentration recorded in the lungs 4 days p.i. was significantly lower than in the control (P≤0.05); however, this was not the case for ST-246. At the same time, both drugs caused a significant reduction in virus titres in mouse lungs 4 days p.i. as compared with the control. NIOCH-14 and ST-246 administered in the same doses did not show significant differences in their anti-smallpox efficacies.

**DISCUSSION**

Most researchers studying the susceptibilities of different mouse lines to search for animal models for monkeypox have aimed at revealing clinical signs of the disease, including the lethal effect (Americo et al., 2010; Stabenow et al., 2010; Earl et al., 2015). It was found that inbred CASA, C57BL/6 stat1−/− and CAST/EiJ mice were highly sensitive to monkeypox virus following i.n. infection (LD_{50} = 100, 47–213 and 680 p.f.u., respectively). Similarly, in experiments involving VARV, other scientists also tried to find sensitive mice following i.n. and intraperitoneal infection (Murti & Shrivastav, 1957; Mayr & Herrlich, 1960; Kaptsova, 1967). However, we did not manage to do it. On the contrary, our research focused on recording not only external signs of the disease, but also the infectious process (by the virus presence in the primary target organs), which significantly reduced the sensitivity threshold of the

**Table 1. Preventive activity of drugs against VARV strain Ind-3a 3 days after i.n. challenge of ICR mice with the dose of 3.7 log_{10} p.f.u. (10 ID_{50})**

<table>
<thead>
<tr>
<th>Index</th>
<th>NIOCH-14</th>
<th>ST-246</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily dose of administered drug (µg g^{-1})</td>
<td>60</td>
<td>60</td>
<td>*</td>
</tr>
<tr>
<td>Number of animals used in experiment</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>VARV concentration in the lungs (log_{10} p.f.u. per lung) of each mouse, 3 days after i.n. challenge</td>
<td>2.4</td>
<td>2.5</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2.3</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td>2.3</td>
<td>4.4</td>
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<tr>
<td></td>
<td>&lt;1.1</td>
<td>&lt;1.1</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>&lt;1.1</td>
<td>&lt;1.1</td>
<td>2.8</td>
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<tr>
<td></td>
<td>&lt;1.1</td>
<td>&lt;1.1</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>&lt;1.1</td>
<td>&lt;1.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Mean virus concentration (log_{10} p.f.u. per lung, M±I_{95}) in mouse lungs 3 days p.i.</td>
<td>2.3±0.5 (n=3)</td>
<td>2.4±0.3 (n=3)</td>
<td>3.6±0.8 (n=7)</td>
</tr>
<tr>
<td>Virus production suppression index (log_{10}) in the lungs of infected mice</td>
<td>1.3</td>
<td>1.2</td>
<td>ND</td>
</tr>
<tr>
<td>Number and percentage (%) of infected mice 3 days p.i.</td>
<td>3 (43)§</td>
<td>3 (43)§</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Coefficient (%) of protection against challenge</td>
<td>57</td>
<td>57</td>
<td>ND</td>
</tr>
</tbody>
</table>

n, Number of animals; M, median value; I_{95}, 95 % confidence level; ND, value was not determined.

*Mice of the control group were administered methylcellulose solution with Tween 80, which was used to suspend NIOCH-14 and ST-246.

†Significant difference from the control (two-tailed Student’s t-test with equal dispersions).

‡Difference between log_{10} of the virus amount in the control and experimental groups of animals.

§Significant difference from the control (Fisher’s exact test, P one-sided ≤0.05).

¶Difference between the percentages of uninfected animals in the experiment and the control. Values <1.1 are lower than the sensitivity threshold (1.1 log_{10} p.f.u. per lung) of the titration method used.
method used to estimate the susceptibility of these animals due to the opportunity of revealing the asymptomatic course of the disease. This allowed us to obtain the indices of susceptibility of ICR and SCID mice to VARV, estimated by the development of an infectious process (Table 3). Moreover, these dose values were 10–1000 000 times lower than those obtained in the animal model for smallpox (M. cynomolgus) and 100 000–1 000 000 times lower than those actually used to challenge these primates with VARV for assessing the efficacies of the developed anti-smallpox drugs (Huggins et al., 2009; Mucker et al., 2013).

Table 2. Preventive activity of drugs against VARV strain Ind-3a 4 days after i.n. challenge of SCID mice with the dose of 4.5 log10 p.f.u. (10 ID50)

<table>
<thead>
<tr>
<th>Index</th>
<th>Mice treated with drugs orally once daily once daily 1 day before and for 3 days p.i.</th>
<th>NIOCH-14</th>
<th>ST-246</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily dose of administered drug (µg g⁻¹)</td>
<td></td>
<td>50</td>
<td>50</td>
<td>*</td>
</tr>
<tr>
<td>Number of animals used in experiment</td>
<td></td>
<td>10</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>VARV amount in the lungs (log10 p.f.u. per lung, M±I95) of each mouse 4 days after i.n. challenge</td>
<td></td>
<td>2.8±2.2</td>
<td>3.1±0.1</td>
<td>3.9±0.8</td>
</tr>
<tr>
<td>Mean virus concentration (log10 p.f.u. per lung, M±I95) in the mouse lungs 4 days p.i.</td>
<td></td>
<td>2.0±0.1</td>
<td>2.9±0.2</td>
<td>3.6±0.9</td>
</tr>
<tr>
<td>Virus production suppression index (log10)† in the lungs of infected mice</td>
<td></td>
<td>3.0±0.1</td>
<td>3.1±0.2</td>
<td>4.3±0.0</td>
</tr>
<tr>
<td>Number and percentage (%) of infected mice 4 days p.i.</td>
<td></td>
<td>2.9±0.6</td>
<td>2.8±0.4</td>
<td>3.8±0.3</td>
</tr>
<tr>
<td>Coefficient (%) of protection</td>
<td></td>
<td>against challenge</td>
<td></td>
<td>3.4±0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;1.1</td>
<td>3.0±0.5</td>
<td>3.7±0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;1.1</td>
<td>3.0±0.4</td>
<td>4.0±0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;1.1</td>
<td>&lt;1.1</td>
<td>4.0±0.9</td>
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<tr>
<td></td>
<td></td>
<td>&lt;1.1</td>
<td>&lt;1.1</td>
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<tr>
<td></td>
<td></td>
<td>&lt;1.1</td>
<td>&lt;1.1</td>
<td></td>
</tr>
</tbody>
</table>

n, Number of animals; M, median value; I95, 95 % confidence level; ND, value was not determined.
* Mice of the control group were administered methylcellulose solution with Tween 80, which was used to suspend NIOCH-14 and ST-246.
† Significant difference from the control (two-tailed Student’s t-test with equal dispersions).
‡ Difference between log10 of the virus amount in the control and experimental groups of animals.
§ Significant difference from the control (Fisher’s exact test, P one-sided ≤0.05).
|| Difference between the percentages of uninfected animals in the experiment and the control. Values <1.1 are lower than the sensitivity threshold (1.1 log10 p.f.u. per lung) of the titration method used.

Table 3. Data on human and animal susceptibilities to variola virus

<table>
<thead>
<tr>
<th>Evaluated indices</th>
<th>Method</th>
<th>ID50 as log10 p.f.u. (M±I95) for macroorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Humans</td>
</tr>
<tr>
<td>Presence of pox-like symptoms</td>
<td>In vivo</td>
<td>&lt;1.0*</td>
</tr>
<tr>
<td>Presence of infectious process</td>
<td>In vivo</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
<td>−0.02±0.23†</td>
</tr>
</tbody>
</table>

ND, No data.
* Value was determined previously (Drozdov et al., 1987).
† Value was obtained from previously presented data (Hahon & Wilson, 1960; Hahon & McGavran, 1961; Noble, 1970; LeDuc & Jahrling, 2001; Jahrling et al., 2004).
‡ Value was determined previously (Zamedyanskaya et al., 2014).
Our expectations based on the information about increased susceptibility to VARV of mice artificially immunosuppressed by Co\textsuperscript{60} radiation treatment (Shafrikova, 1970) were not justified in experiments on immunodeficient SCID mice, which had neither clinical signs of the disease nor increased (as compared with ICR mice) maximal concentration of the virus in the lungs. Moreover, ID\textsubscript{50} value in SCID mice was, though insignificantly, higher than in mice ICR. This discrepancy of the obtained results may be due to the difference in immunodeficiency levels of SCID mice with severe combined immunodeficiency (deficiency of T- and B-lymphocytes and immunoglobulins) (Belizário, 2009) and Co\textsuperscript{60}-treated mice.

Noteworthy is the fact that, in addition to the brain and respiratory tract, we could find VARV in virtually none of the samples from mice during the entire period of observation, indicating the absence or extremely low sensitivities of cells of many organs of experimental animals to the virus. This circumstance resulted in limited development of an infectious process in mice which steadily affected only the primary target organs of respiratory tract. However, the pathogen was twice found in very low concentrations in the brains of ICR mice only. The reduction of VARV concentration to undetectable levels in the lungs and nasal septum of ICR mice 10 days p.i. could be due to the influence of the factors of immune response of these animals.

The fact of the virus replication in the respiratory tract of ICR and SCID mice following infection with VARV is to a certain extent consistent with that in humans and the known animal model (Hahon & Wilson, 1960; Sarkar et al., 1973a, b; Jahrling et al., 2004; Marennikova & Shchelkunov, 2005). Researchers found the maximal virus concentrations in pharyngeal swabs of humans \[3–5 \log_{10} \text{ pock forming units ml}^{-1}\] (Sarkar et al., 1973a) and \textit{M. cynomolgus} \[2.0–4.5 \log_{10} \text{ p.f.u. ml}^{-1}\] (Jahrling et al., 2004), as well as in homogenates of nostrils and nasopharynx of \textit{M. cynomolgus} \[2.0–3.3 \log_{10} \text{ pock forming units ml}^{-1}\] (Hahon & Wilson, 1960), similar to our data on the virus accumulation in the noses of ICR and SCID mice. However, the duration of the virus presence in the upper respiratory tract of primates and humans (Hahon & Wilson, 1960; Marennikova & Shchelkunov, 2005) was considerably longer (\[\geq 11\] days) than in i.n. challenged mice (3–6 days). The viral titres \[3.8–5.5 \log_{10} \text{ pock forming units ml}^{-1}\] in the lungs of the model animal (\textit{M. cynomolgus}) following aerosol challenge with the dose of \[4.7 \log_{10} \text{ pock forming units ml}^{-1}\] were similar (Hahon & Wilson, 1960) to those obtained by us in ICR and SCID mice challenged i.n. with similar doses of \[4.2\] and \[5.2 \log_{10} \text{p.f.u.}\], respectively. There were no significant differences in the duration of infectious processes in the lungs of primates and mice (8–11 days). It is also interesting to note that in experiments involving another representative of orthopoxviruses (ectromelia virus strain Moscow) for which the range of sensitive animals is restricted exclusively to mice, a number of researchers infected groups of control A/NCr mice i.n. (10 and 100 LD\textsubscript{50}) to assess ST-246 efficacy (Yang et al., 2005; Quenelle et al., 2007). The maximum values of the virus concentration in lung homogenates (4.3 and 4.7–5.5 \log_{10} \text{ ml}^{-1}) similar to those obtained by us in ICR and SCID mice following i.n. challenge with 30 and 50 ID\textsubscript{50} of VARV, were recorded (Fig. 2a, b).

Unlike man and the animal model (\textit{M. cynomolgus}), in which generalized disease course with the corresponding symptoms was observed following respiratory infection, the infectious process in ICR and SCID mice was asymptomatic and was restricted mainly to primary respiratory organs. This excludes the possibility of using these animals to evaluate the therapeutic efficacy of the developed anti-smallpox drugs, but supports the possibility of their use to study their preventive (extreme preventive) activity. This is due to the fact that preventive (extreme preventive) administration of drugs aims at the virus control in primary target organs, which are located in human and mouse respiratory tracts following respiratory infection.

The conducted comparative analysis of literature regarding pathological changes in humans and the animal model (\textit{M. cynomolgus}) after challenge with VARV via respiratory tract (Councilman et al., 1904; Lillie, 1930; Bras, 1952; LeDuc & Jahrling, 2001; Cann et al., 2013) and the results of our studies in i.n. challenged ICR and SCID mice evidenced their great similarity regarding the inflammatory destructive effect on respiratory tract organs.

We attempted to identify the primary target cells for VARV when carrying out electron microscopy of organs and tissues of i.n. challenged ICR and SCID mice. The main focus was placed on the study of the organs of the mouse respiratory tract given that these organs are the main and almost the only primary site of the virus reproduction in these animals. However, these studies were unsuccessful, which could most likely be explained by insufficiently high concentrations of the virus in the examined organs and tissues, that in none of the cases exceeded \[5 \log_{10} \text{ p.f.u. ml}^{-1}\] of homogenate. However, signs of VARV reproduction in these cells were revealed with virological and electron microscopic methods in experiments \textit{in vitro} using the cells of the mononuclear phagocyte system (spleen monocytes–macrophages) obtained from ICR mice (Figs 3 and 4k, l). The results of these experiments can be extrapolated to SCID mice, given the fact that severe combined immunodeficiency created in these mice affected only the T- and B-cell component and did not cause any changes in other cells of the organism, including the mononuclear phagocyte (macrophage) systems. VARV accumulation was revealed following titration of homogenates of trachea and nasal septum with mucosa in i.n. challenged ICR and SCID mice at 2 and 3 days p.i. (Fig. 2), which together with the data on inflammatory and destructive changes in respiratory tract mucosa of these animals indicated the involvement of epithelial cells in the infectious process. In the scientific literature, VARV replication in cells of the mononuclear phagocyte (macrophage) system and epithelial cells of the respiratory tract was also noted in the animal model for smallpox following aerosol and intravenous (i.v.) challenge (Hahon & Wilson, 1960; Jahrling
et al., 2004; Wahl-Jensen et al., 2011). At the same time, some scientists conducting research involving the use of another representative of orthopoxviruses (vaccinia virus) on mouse monocyte–macrophage cell culture (I774.G8 cell line) observed an abortive infection with the infectious process blocked at an early stage (Humlová et al., 2002). This discrepancy between these findings and our results obtained in mouse monocytes–macrophages can be explained by differences in the types of viruses and cell cultures used.

Almost all indices of interaction of ICR and SCID mice with VARV, under criteria associated with the search for animal models to be used for assessment of only prophylactic (emergency and prophylactic) efficacy of anti-smallpox drugs, are consistent with or similar to those in humans and the known animal model, cynomolgus macaque. Primates (M. cynomolgus) are currently used as animal models for variola only to evaluate therapeutic activities of drugs tested in experiments on i.v. injection of VARV in huge doses (8 and 9 \( \log_{10} \) p.f.u.) (Huggins et al., 2009; Mucker et al., 2013). This is due to the fact that these animals, to a certain extent, reproduce only the second and the third stages of the infectious process observed for smallpox in humans: active delivery (and replication) of the virus via the haematogenous route to secondary target organs (Stage 2) and the virus replication in secondary target organs (Stage 3). Pathological changes occurring in secondary target organs of these primates were similar to those observed in humans who died of smallpox. On the contrary, ICR and SCID mice reproduce only the first stage of the infectious process (virus replication and pathomorphological changes in respiratory organs; virus replication in the corresponding target cells), and do not reproduce the second and third stages. This excludes the possibility of using these animals to evaluate the therapeutic efficacy of the developed anti-smallpox drugs, but creates the possibility of their use to study drug preventive (extreme preventive) activity, which is not possible with intravenously challenged primates. In this regard, the studies on the antiviral activity of NIOCH-14 being developed in Russia in comparison with the US drug ST-246 were conducted in order to verify the adequacy of results of using such models. The study of the prophylactic activity levels of these drugs in ICR and SCID mice using VARV confirmed the presence of a positive effect observed previously by us and many researchers (Nalca et al., 2008; Jordan et al., 2009; Smith et al., 2009, 2011; Sergeev et al., 2015a, b), which indicates the adequacy of animal models used by us for this purpose, including immunodeficient SCID mice reflecting the expressed immunosuppressive condition in humans.

Thus, despite the fact that the ICR and SCID mice challenged i.n. with VARV did not reproduce the clinical picture of a pox-like disease, their susceptibilities to this virus were relatively high (by recording the pathogen presence in the lungs), and they simulated the first component of the smallpox infectious process in humans (or the animal model, cynomolgus macaque), including the virus replication and pathological changes in respiratory tract and the types of the primary target cells, as well as adequately demonstrated that the tested drugs produced antiviral effects following i.n. challenge with VARV. All this makes possible the actual use of these animals in experiments involving the use of VARV to assess the prophylactic (emergency and prophylactic) action of the developed anti-smallpox drugs. The study of the therapeutic activity levels of anti-smallpox drugs can be carried out using VARV in the previously known model (cynomolgus macaque).

**METHODS**

All experiments involving the use of live VARV were conducted at FBRI SRC VB Vector (Vector), in a maximum containment facility (BSL-4) using insulating pneumatic suits.

**Animals.** The studies involved the use of outbred male and female 10–14 day ICR mice (weighing 8–10 g) obtained from Vector’s nursery. These animals are similar to HA(ICR) mice from Jackson laboratory and originated from Swiss Webster mice previously purchased by the Soviet Union. The study also involved male and female 18–21 day immunodeficient SCID mice (Hairless Outbred SHO(Rb)-H- mouse, weighing 12–14 g) obtained from the SPF-vivarium of the Institute of Cytology and Genetics (RFMEF61914x0005 and RFMEF62114x0010) where this line came from Charles River Laboratories in 2012.

Experimental animals were kept on a standard diet with enough water in accordance with the veterinary legislation and requirements for humane animal care and use in experimental studies (National Research Council, 2011). Research and manipulations on animals were conducted with the approval of Vector’s Bioethics Committee no. 1-01-2014 dated January 28, 2014.

**Cell culture.** A continuous Vero cell culture obtained from the Cell Culture Collection of Vector was used to produce a virus-containing suspension and to titrate various samples. Vero cell monolayer was prepared in the cultivation supporting one for the virus cultivation.

**Viruses.** The study involved the use of VARV strain Ind-3a from the State Collection of infectious viral and rickettsial agents of Vector. Using this strain, a virus-containing suspension having bioconcentrations of \( 6.7 \pm 0.1 \log_{10} \) p.f.u. ml\(^{-1} \) was prepared in the cultivation medium. Virus-containing material was packaged in individual vials and stored at \(-70 ^\circ C\).

**Viruses infecting doses for mice and preparation of samples.** Upon anaesthesia with inhaled isoflurane, the mice were challenged i.n. with a total of 0.03 ml of virus-containing fluid to both nostrils. The animals were challenged with a single dose of 5.2 log\(_{10}\) p.f.u. using six animals per dose in experiments to assess the sensitivity of ICR and SCID mice to VARV based on the detection of the disease clinical signs. To study the dynamics of the virus accumulation in the lungs, ICR and SCID mice were challenged with infectious doses of 4.2 and 5.2 log\(_{10}\) p.f.u., respectively, and (after euthanasia by cervical dislocation) their whole lungs were taken 1, 2, 3, 4, 5 and 7 days p.i. from each individual animal using three mice per each time point, and preparing 10 % homogenates by mechanical destruction with a pestle in a mortar with river sand.

In experiments to assess the sensitivity of animals to VARV based on the virus detection in the lungs, ICR and SCID mice were challenged with
the virus doses (1.2–5.2 log_{10} p.f.u.) diluted with 1 log_{10} steps using six and four animals per dose, respectively, and 3 and 4 days p.i. respectively, whole lungs were taken after euthanasia to prepare homogenates from each animal. Data on the presence/absence of VARV in the lungs 3 and 4 days p.i. were used to calculate ID_{50} of VARV for mice.

In experiments to study the dynamics of the virus accumulation in organs, tissues and serum, ICR mice were challenged i.n. with a single dose of the virus (4.2 log_{10} p.f.u.) followed by collecting samples 1, 2, 3, 4, 5, 6, 7 and 10 days p.i. and using eight animals per each time point. In similar experiments involving the use of SCID mice, the latter were also challenged with a single dose of 5.2 log_{10} p.f.u. followed by collecting samples 1, 2, 3, 4, 5 and 7 days p.i. and using eight animals per each time point. Before euthanasia, blood samples were collected from the retrotrobiatal venous sinus of ICR and SCID mice and used to prepare serum and a clot of formed elements by centrifugation for the subsequent preparation of homogenate. Moreover, after euthanasia, samples (nasal septum with mucosa, brain, trachea, bifarication lymph nodes, lungs, liver, spleen, pancreas, duodenum and kidneys) were taken from infected animals. Organs and tissues, including clots of blood cells from each of four individual animals collected at the corresponding time points, were homogenized for the subsequent virological investigation.

In experiments to study the dynamics of the virus accumulation in mice, samples of organs and tissues were collected simultaneously (blood cells, nasal septum with mucosa, brain, trachea, bifarication lymph nodes, lungs, liver, spleen, pancreas, duodenum, mesenteric lymph nodes, kidneys, adrenals and skin pieces) from four ICR and SCID mice per each point to study pathological changes. To study drug efficacy, ICR and SCID mice were challenged with the doses of 3.7 and 4.5 log_{10} p.f.u., respectively, and 3 and 4 days p.i., respectively, their whole lungs were taken after euthanasia to prepare homogenates from each individual animal.

Production of primary monolayer cultures of mouse spleen monocyte–macrophage and infecting them with VARV. To obtain SMMMPC, the spleen of ICR mice euthanized by cervical dislocation was homogenized in a test-tube with a glass pestle and washed with RPMI 1640 medium (Vector). One microlitre of cell suspension prepared in growth medium (RPMI 1640 medium containing 10 % FBS and 80 μg gentamicin ml^{-1}) was placed into each well of a 24-well culture plate and incubated in an atmosphere containing 5 % CO2 at the temperature of 37 °C for 24 h. After washing twice with RPMI 1640 medium, the remaining adherent monocytes–macrophages were infected with VARV at an m.o.i. of 0.003 and 0.017 p.f.u. per cell and cultivated for 3 days in the same medium though supplemented with 2 % FBS and 80 μg ml^{-1} of gentamicin (supporting medium) under other conditions described above. At each time point 1, 24, 48, 72, 144 and 168 h p.i. four samples were taken from four wells after triple freezing and thawing to determine the biological concentration of the virus. VARV-infected cell monolayers were washed and removed with a rubber crusher, and samples were prepared for electron microscopic examination.

As a negative control, we used a suspension of cell debris produced by triple freezing and thawing of SMMMPC (by cell number) in supporting medium followed by testing for the absence of viable cells using light microscopy and inoculation onto a culture plate.

Administration of chemical compounds to mice challenged with VARV. The study involved the use of chemical compounds with known anti-orthopoxvirus activity levels (Yang et al., 2005; Mucker et al., 2013; Sergeev et al., 2015a, b); NIOCH-14 [7-N’-[4-(trifluoromethyl benzoyl)-hydrazinocarbonyl]-tricyclo[3.2.2.0^{2,6}][6]non-8-en-6-carboxylic acid] and ST-246 [4-(trifluoromethyl)-N-[3a,4,4a,5,5a,6,6a-octahydro-1, 3-dioxo-4,6-etheno-cycloprop[f]isoindol-2-(1H-γ)benzamide]] synthesized for the study according to a described technique (Jordan et al., 2005). Both these drugs had similar mechanisms of antiviral action, which were associated with blocking the process of formation of the virion envelope in infected cells negatively influencing the formation of a pathogenetically significant extracellular enveloped virus (Jordan et al., 2010; Shishkina et al., 2015).

ICR mice were given orally 0.2 ml of suspension of these compounds at the dose of 60 μg (g body weight)^{-1} once daily 1 day before challenge, 2 h, 1 and 2 days after challenge and SCID mice received the dose of 50 μg (g body weight)^{-1} once daily 1 day before challenge, 2 h, 1, 2 and 3 days after challenge with VARV. As a placebo, we injected 0.2 ml of a solution containing 0.75 % methylcellulose and 1 % Tween-80 used to prepare NIOCH and ST-246 suspensions.

Virological analysis of samples. Viable virus concentrations in sera and organ and tissue homogenates of infected animals were determined with the traditional method of plaque quantification by inoculating serial dilutions of samples into Vero cell monolayer (Leparc-Goffart et al., 2005). The minimum amount of the virus that could be detected by the titration method used was 0.4 p.f.u. ml^{-1} and 1.1 log_{10} p.f.u. per lung for organ and tissue homogenates, and 0.4 log_{10} p.f.u. ml^{-1} and 1.1 log_{10} p.f.u. per lung for serum, while for samples obtained from the virus cultivation in SMMMPC and the control, it was 0.7 log_{10} p.f.u. ml^{-1}.

Electron microscopy. Sample preparation and recording of the results of the study were also conducted as described previously (Sergeev et al., 2015a).

Statistical treatment of results. Statistical treatment of results was carried out with standard methods (Zaks, 1976) using the software package Statistica 6.0 (StatSoft 1984–2001) with assessment of significant differences for P ≤ 0.05 (I_{50}). ID_{50} doses were calculated using the Spearman–Karber formula. The proportions of animals with the virus in the lungs 3 or 4 days p.i. were taken into account in each group of mice infected with different doses of VARV. In experiments to evaluate the therapeutic and prophylactic effects of drugs, the comparison of the proportions of infected animals in groups was conducted by the χ^2 test, while the U-Mann–Whitney test and the Student’s t-test were used to compare VARV titres in mouse lungs (Zaks, 1976).

REFERENCES


