INACTIVATION OF RICKETTSIAE

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Received: May 24, 2013; Accepted: May 29, 2013

A reliable and complete inactivation is an indispensable premise for any concentration of rickettsiae or for the development of diagnostic strategies based on their antigens. This study deals with the testing of methods to inactivate rickettsiae.

Rickettsia honei was used as a model organism. The inactivating potency of formalin, Qiagen® *antiviral lysozyme* (AVL) buffer, heating to 56 °C, and β -propiolactone was analyzed in cell culture.

The inactivation limits for rickettsiae were 0.1% formalin about 10 min, Qiagen AVL buffer about 5 min, 56 °C about 5 min, 0.125% β -propiolactone about 1 h, and 0.0125% β -propiolactone overnight. The interpretation was limited by cytotoxic effects of the inactivation procedures and by the culturally achievable rickettsial density in the cell culture supernatants that were used for the inactivation experiments.

Reliable modes of inactivation were identified, allowing for the secure handling of rickettsial antigens for diagnostic purposes.

Keywords: rickettsiae, inactivation, formalin, AVL, heat, β -propiolactone

Introduction

A successful inactivation procedure is an important precondition for the secure handling of rickettsiae. Severe laboratory infections with rickettsiae have been described [1, 2]. A lot of different inactivation procedures are performed in various laboratories. However, comparative studies on the inactivation of rickettsiae are missing.

Different formalin-based inactivation schemes have been described so far. Reports on inactivation procedures based on 0.01% formalin overnight [3] and for 72 h [4] can be found in the scientific literature of the 1970s. Inactivation regimes using the higher concentration of 0.1% formalin are favored at present [5]. Paraformaldehyde in a concentration of 3.5% about 20 min has been described to inactivate rickettsiae as well [6]. About 1% sodium dodecyl sulfate (SDS) was used but it destroys the rickettsial cellular integrity [7]. An inactivation method including the treatment with acetone about 10 min is particularly well-suited for immuno-fluorescence [6]. The diving of the samples in 70% alcohol about a few minutes was previously performed as well [8]. Another group reported three times washing in 70% alcohol for 10 min followed by an additional washing step in sodium hypochlorite for further 10 min [9]. The use of iodinated alcohol (consist-

ing of 12 ml iodine and 6 ml ethylene glycol per liter of 70% alcohol) was also suggested [10]. Inactivation of rickettsiae in samples using 200 krad Gamma-radiation was described in the 1990s [11].

This study evaluated a set of selected inactivation procedures. Formalin was chosen because of its potency to widely preserve the integrity of rickettsial antigens. The inactivating effect of *antiviral lysozyme* (AVL) buffer (Qiagen, Hilden, Germany) was evaluated because of its suitability to inactivate rickettsiae prior to polymerase chain reaction (PCR) analyses. A heat inactivation procedure with temperatures about 56 °C that is efficient for the inactivation of many viruses [12] was tested as well. In addition, the inactivating potency of β -propiolactone (BPL) was analyzed. This agent acts on DNA level and is not harmful for rickettsial antigens [13].

Materials and methods

Model organism

Tick-transmitted bio-safety level (BSL) 2 *Rickettsia (R.) honei* (ATCC VR-1472), the causative pathogen of Flinders Island spotted fever in Australia [1], was chosen

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Fig. 1. Cytopathic effect induced by *R. honei* in the Vero E6 cell mono-layer

as a model organism. This agent is suitable for inactivation trials because it can be grown in Vero E6 cell mono-layers to high titers where it induces a cytopathic effect (Fig. 1). The cytopathic effect in the cellular mono-layer usually appears between the third and the fifth day of incubation. Further, *R. honei* can be conveniently grown outside a BSL 3 laboratory. Rickettsial growth can be confirmed with semi-quantitative real-time PCR targeting the rickettsial *gltA* gene as described [14].

able inactivation. A positive control was not treated with formalin. A negative control contained only cell culture medium. A dilution series including the six dilution steps $1:10^0$ to $1:10^5$ was pipetted after the inactivation procedures in 1.5-ml cups (Eppendorf, Hamburg, Germany). Volumes of 1 ml each were achieved by dilution in minimal essential medium (MEM) (Gibco®, Invitrogen Ltd., Karlsruhe, Germany). A new pipet tip was used for every dilution step. The six wells of six-well plates containing

Inactivation procedures

R. honei was grown in cell culture bottles on confluent Vero E6 cell mono-layers. After the complete destruction of the mono-layer due to the cytopathic effects of *R. honei* after 4–5 days of incubation, the supernatant was used for several inactivation procedures.

Inactivation trials with formalin

The rickettsiae containing cell culture supernatant was treated with concentrations of 1%, 0.1%, 0.01%, and 0.001% formalin about 1 h at room temperature to determine the minimum formalin concentration that is required for a reliable

Fig. 2. Proof of the replication of rickettsial DNA due to growth in cell culture by semi-quantitative real-time PCR by the reduction of the cycle threshold (Ct) values for six decadic logarithmic dilution steps ($1:10^0$ – $1:10^5$), measurement at the days 0, 3, and 5

confluent mono-layers of Vero E6 cells were inoculated with 300 µl of the 6 dilutions ($1:10^0$ to $1:10^5$) in decreasing concentrations. The potentially inactivated samples were incubated in the inoculated wells for 1 h at 37 °C and 5% CO₂. Afterwards, the inoculums were removed, and the wells were refilled with MEM enriched with non essential amino acids (NEA) and 3% foetal calf serum (FCS) (Gibco, Invitrogen). The cell culture plates were incubated at 37 °C and 5% CO₂ and analyzed for cytopathic effects within the first hour after refilling with medium and at the days 3 and 5 of incubation.

Volumes of 200 µl were taken from the cell culture supernatant for semi-quantitative *gltA* gene real-time PCR analyses at the days 0, 3, and 5 after infection to confirm or exclude the replication of rickettsial DNA (Fig. 2). The correlation between the cytopathic effect and rickettsial growth in cell culture was confirmed by this procedure.

After the demonstration that 0.1% formalin is the lowest formalin concentration that guarantees a reliable inactivation within 60 min, a time kinetic of the inactivating effects of this formalin concentration was generated. A dilution of 1:100 was performed after 5, 10, 15, 20, 30, 45, and 60 min to neutralize the inactivating effects of the formalin. Subsequently, a dilution series including the 6 dilution steps $1:10^2$ to $1:10^7$ was incubated on confluent mono-layers of Vero E6 cells in six-well plates as described above. All samples were inoculated on Vero E6 cells 60 min after the start of the inactivation process in order to assure equal conditions concerning the storage time of *R. honei* outside the incubator.

Incubation and growth conditions in the six-well plates were as described above. Positive and negative controls were included. Cytopathic effects on the cell mono-layers were evaluated at the day of inoculation as well as at the days 4 and 7 after the beginning of incubation.

Inactivation trials with Qiagen® AVL buffer

Qiagen® AVL buffer must not be diluted to ensure its inactivating effects according to the manufacturer's instructions. An in-house preliminary test demonstrated that a 1:100 dilution of this buffer neutralizes its potency to inactivate rickettsiae. That is why we abstained from the exact definition of the minimum neutralizing concentration of AVL buffer. Instead of that a time kinetic of the inactivating effect of AVL was determined. A dilution to 1:100 after 5, 10, 15, 20, 30, 45, and 60 min was performed to neutralize the inactivating effect of the agent during this experiment. The inactivation process was performed at room temperature. The trial was accomplished following the same protocol that is described above for the inactivation with formalin. Positive and negative controls were included. Cytopathic effects were evaluated directly after the inoculation of the samples and at the days 4 and 7 of incubation.

Inactivation trials with temperature about 56 °C

At first, the inactivating effects of heating to 56 °C about 30 min on *R. honei* were analyzed. Subsequently, a time kinetic of inactivation about 5, 10, 15, 20, 25, and 30 min was generated. The inactivation of the rickettsiae-containing cell culture supernatants with 56 °C was performed in 1.5-ml Eppendorf cups that were completely dipped under water. Such a complete dipping is unavoidable to ensure a constant temperature in the whole sample tube. A dilution series including the 6 dilution steps 10^0 to 10^5 was pipetted after the inactivation procedure. The further procedures including incubation and growth in six-well plates were identical with the protocols for the formalin or Qiagen® AVL buffer-based inactivation. Positive and negative controls were included. Cytopathic effects were analyzed directly after the inoculation and at the days 4 and 7 of incubation.

Inactivation trials with β-propiolactone

Inactivation procedures with β-propiolactone require concentrations between 0.125% and 0.25% to ensure a secure inactivating effect within a short period of time [13]. β-propiolactone is very susceptible to hydrolysis according to the manufacturer's instructions. One hour was chosen as the minimum inactivation time without an inactivation kinetic to ensure a wide hydrolytic cleavage of the very toxic agent to the non-toxic hydracrylic acid. The inactivation procedure was performed at room temperature. The probability of disturbances of the antigenicity of the rickettsiae was low because β-propiolactone is known to act mainly on DNA level [13]. A dilution series including the six dilution steps 10^0 to 10^5 was created after the inactivation process. Incubation and growth in six-well plates was performed according to the protocols described above. Positive and negative controls were included as well. Cytopathic effects were analyzed directly after the inoculation and at the days 5 and 7 of incubation.

The limits of the inactivating efficacy of β-propiolactone and a possible decrease of its cytotoxic effects on cell culture due to proceeding hydrolysis were tested afterwards. In order to do so, concentrations of the agent of 0.125%, 0.0125%, and 0.00125% were incubated with the rickettsiae about 1 h or overnight, respectively. Incubation about 1 h was performed at room temperature, and incubation overnight at 4 °C in the fridge. The further procedure was identical with the scheme described above. Positive and negative controls were included. Cytopathic effects were analyzed directly after the inoculation and at the days 4 and 7 after incubation.

Interpretation of the results

The tissue culture infecting doses (TCID) of the rickettsiae-containing medium that was used for the inactivation

Fig. 3. Effect of different inactivation methods, 56 °C = heat inactivation at 56 °C, c.t. = cytotoxic effect because of the inactivating agent, i.e. = inactivating effect, p.c. = positive control, r.g. = rickettsial growth, o.n. = over the night, ' = minutes

Fig. 4. Time kinetic of inactivation with 0.1% formalin, AVL, and heat inactivation at 56 °C; 56 °C = heat inactivation at 56 °C, P.c. = positive control

experiments were identified using decadic logarithmic dilutions and consecutive inoculation in Vero E6 cell culture wells of the positive controls. Rickettsial growth was visualized by cytopathic effects. A determination of the reduction of the number of TCIDs due to the inactivation procedures was possible by comparison with the positive controls. The cell culture supernatants that were used for the experiments contained 10^5 – 10^6 TCIDs. Cytopathic effects in Vero E6 cell culture did not allow for growth to higher titers, a limitation of the experimental setting.

Results

Inactivating effects of formalin

An inactivating effect on *R. honei* about three and four decadic logarithmic steps at an inactivation term of 60 min could be demonstrated for formalin concentrations of 1% and 0.1%, respectively. The positive control revealed that the inactivated initial rickettsial doses were about 10^6 TCID each time. However, the mentioned formalin

concentrations led to a cytotoxic effect on Vero E6 mono-layers down to a dilution of 1 : 100 to 1 : 10, respectively. Formalin concentrations of 0.01% or lower did not show any potency to inactivate rickettsiae within the given time of 60 min (Fig. 3).

We were able to demonstrate that the inactivating effects of 0.1% formalin can be neutralized by a 1 : 100 dilution in medium. A time kinetic of the inactivation revealed that 5 min in 0.1% formalin is not yet enough to securely inactivate rickettsiae. However, an inactivating effect about at least three decadic logarithmic steps was proven already after 10 min. The initial rickettsial dose in this experiment was 10^5 TCIDs. However, the first two decadic logarithmic steps could not be interpreted because of the 1:100 dilutions that were necessary to neutralize the cytotoxic effects of the used formalin (Fig. 4).

Inactivating effects of Qiagen® AVL buffer

Qiagen® AVL buffer led to cytotoxic effects in Vero E6 mono-layers about three decadic logarithmic steps. That is why an inactivating effect about only two decadic logarithmic steps could be demonstrated for this agent while an initial rickettsial dose of 10^5 TCIDs was demonstrated in the positive control wells for this experiment (Fig. 3).

A time kinetic of the inactivation proved the inactivating effect of AVL buffer on rickettsial agents already after 5 min (Fig. 4).

Inactivating effects of temperature about 56 °C

Heating to 56 °C about 30 min led to an inactivating effect on rickettsiae for the complete 10^6 TCIDs (Fig. 3). A time kinetic of inactivation showed the inactivating effect already after 5 min (Fig. 4).

Inactivating effects of β -propiolactone

The inactivating effect of 0.25% β -propiolactone about 60 min could be demonstrated about two decadic logarithmic steps. An initial rickettsial dose of 10^4 TCID was used for this experimental setting; however, the Vero E6 mono-layer was destroyed down to a dilution of 1 : 10 by the cytotoxic effects of the inactivating agent. An initial rickettsial dose of 10^6 TCID could be used for the analyses of 0.125%, 0.0125%, and 0.00125% β -propiolactone about 1 h and over the night. The inactivating effect of BPL about the term of 1 h could be shown for four decadic logarithmic steps while two dilution steps were not interpretable due to the cytotoxic effects of the inactivating agent. The same BPL concentration led to an inactivation about five decadic logarithmic steps overnight at 4 °C. Only one dilution step was destroyed by the cytotoxic effects of BPL due to an increased hydrolysis of BPL overnight. A BPL concentration of 0.0125% was not reliably

inactivating. Though there was still a reduction of infectivity about three decadic logarithmic steps, rickettsial growth was still detectable down to a dilution of 1 : 100. One dilution step was not interpretable due to the cytotoxic effects of BPL. The hydrolysis of 0.0125% BPL overnight ensured that no remaining cytotoxic effect was visible in the cell culture after the inoculation procedure during the next day. This BPL concentration combined with an inactivation term overnight led to an inactivating effect on *R. honei* about six decadic logarithmic dilution steps, so no rickettsial growth was detectable any more in Vero E6 cell culture. No inactivating effect on rickettsial organisms and no cytotoxic effects were induced by a BPL concentration of 0.00125% about 1 h and over the night (Fig. 3).

Discussion

The experimental setting identified procedures that reliably inactivate *R. honei*. As far as cytotoxic effects of the inactivation procedures allowed for an interpretation, there was an inactivation of the rickettsial growth induced by 0.1% formalin about 10 min, Qiagen® AVL buffer about 5 min, heating to 56 °C about 5 min, 0.125% β -propiolactone about 1 h, and 0.0125% β -propiolactone overnight. However, these are only minimum requirements. As a safety margin, 0.1% formalin about 60 min, Qiagen® AVL buffer about 60 min, 56 °C about 30 min or 0.125% β -propiolactone overnight might be considered, because cytotoxic effects of some inactivation schemes allowed for analyses of only few dilution steps.

The inactivation procedures with formalin, heat, and β -propiolactone were chosen for this analysis because they do not destroy the structural integrity of rickettsiae and allow further purification steps to concentrate the inactivated rickettsiae for diagnostic purposes. AVL buffer is not suitable for this use because of its detergent effects.

Formalin was selected because it is widely used for the inactivation of microbes with little harm to their antigenicity [3–5]. Major advantages in the handling of formalin comprise its good manageability and stability when stored at room temperature. However, disadvantages include harmful effects on lipo-polysaccharides and potential influences on the antigenicity of rickettsial proteins due to changes of their tertiary structure.

Heat inactivation and inactivation by β -propiolactone require a number of premises that have to be considered. The handling of BPL might be harmful due to its carcinogenic effects as demonstrated in mice [12]. Working with BPL under the hood using nitrile gloves should be considered. However, hydrolyzed metabolites of BPL do not show these carcinogenic effects and hydrolysis occurs already during the inactivation process [12]. The metabolite 3-hydroxy-propanoic acid can even be degraded by mammalian cells [13].

Heat inactivation procedures require an equally high temperature in the whole sample tube [12], e.g., by diving after sealing the tube. Insufficient temperature maxima at

the top of the sample tubes during the inactivation process in the heating device led to a loss of the inactivating efficacy of this procedure in prior experiments (data not shown).

Heat and BPL may influence the tertiary structure of proteins and their antigenicity. In particular, heat leads to an enhanced coagulation and precipitation of proteins [12]. In contrast, the mobility of proteins in SDS–polyacrylamid gels is not affected by BPL treatment [13].

None of the mentioned inactivation procedures is completely free of side effects on the antigenicity of inactivated microbes. The most suitable inactivation method has to be chosen depending on the intended use of the inactivated antigen. The identification of the most suitable inactivation procedure for different diagnostic applications remains an aim for further investigations.

Acknowledgements

The authors are grateful to R. Terzioglu for performing the PCR analyses.

References

1. Parola P, Paddock CD, Raoult D: Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clin Microbiol Rev* 18, 719–756 (2005)
2. Raoult D, Roux V: Rickettsioses as paradigms of new or emerging infectious diseases. *Clin Microbiol Rev* 10, 694–719 (1997)
3. Palmer EL, Mallavia LP, Tzianabos T, Objeski JF: Electron microscopy of the cell wall of *Rickettsia prowazekii*. *J Bacteriol* 118, 1158–1166 (1974)
4. Dasch GA, Weiss E: Characterization of the Madrid E strain of *Rickettsia prowazekii* purified by renografin density gradient centrifugation. *Infect Immun* 15, 280–286 (1977)
5. La Scola B, Rydkina L, Ndiokubwayo JB, Vene S, Raoult D: Serological differentiation of murine typhus and epidemic typhus using cross-adsorption and western blotting. *Clin Diagn Lab Immunol* 7, 612–616 (2000)
6. Marzinez JJ, Cossart P: Early signaling events involved in the entry of *Rickettsia conorii* into mammalian cells. *J Cell Sci* 117, 5097–5106 (2004)
7. Ishikura M, Fujita H, Ando S, Matsuura K, Watanabe M: Phylogenetic analysis of spotted fever group rickettsiae isolated from ticks in Japan. *Microbiol Immunol* 46, 241–247 (2002)
8. Nilsson K, Lindquist O, Liu AJ, Jaenson TGT, Friman GR, Pahlson C: *Rickettsia helvetica* in *Ixodes ricinus* ticks in Sweden. *J Clin Microbiol* 37, 400–403 (1999)
9. Pornwiroon W, Pourciau WW, Foil LD, Macaluso KR: *Rickettsia felis* from cat fleas: isolation and culture in a tick-derived cell line. *Appl Environ Microbiol* 72, 5589–5595 (2006)
10. Raoult D, La Scola B, Enea M, Fournier PE, Roux V, Fenollar F, Galvao MAM, de Lamballerie X: A flea-associated rickettsia pathogenic for humans. *Emerg Infect Dis* 7, 73–81 (2001)
11. Kelly DJ, Chan CT, Paxton H, Thompson K, Howard R, Dasch GA: Comparative evaluation of a commercial enzyme immunoassay for the detection of human antibody to *Rickettsia typhi*. *Clin Diagn Lab Immunol* 2, 356–360 (1995)
12. Ball MJ: Effect of two disinfectant treatments on laboratory analyses. *J R Soc Med* 80, 482–484 (1987)
13. Brown A, Consigli A, Zabielski J, Weil R: Effect of beta-propiolactone inactivation of polyoma virus on viral functions. *J Virol* 14, 840–845 (1974)
14. Wölfel R, Terzioglu R, Kiessling J, Wilhelm S, Essbauer S, Pfeffer M, Dobler G: *Rickettsia* spp. in *Ixodes ricinus* ticks in Bavaria, Germany. *Ann N Y Acad Sci* 1078, 509–511 (2006)