In vivo and in vitro characterization of two camelpoxvirus isolates with decreased virulence

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Key words
Dromedary - Camelus dromedarius - Camelus bactrianus - Orthopoxvirus - In vitro experimentation - In vivo experimentation - Vaccine.

Summary
Two camelpoxvirus (CPV) strains isolated from camels with generalized skin disease were serially passaged on Vero cells. Various phenotypic properties were investigated in vitro and in vivo and compared with those of the corresponding wildtype strains. In many aspects no differences were observed. However, in a mouse model both passaged strains proved to be highly attenuated. In addition, both strains failed to replicate in a cell line derived from camel skin cells. Comparison of physical maps established for enzymes HindIII and Xhol revealed deletions accounting for a total of 22 kbp in one attenuated strain. In the second strain only minor alterations were noted.

INTRODUCTION

Today almost 20 million camels (Camelus dromedarius and Camelus bactrianus) are kept in Africa, Asia, India and the Middle East for milk, meat and wool production, transport or racing (18). As the interest in husbandry and diseases of camels has increased, camelpox in particular has received attention (20). It is caused by the so-called camelpoxvirus (CPV) which represents a separate species within the genus Orthopoxvirus (19). Mainly camel calves are affected and develop a proliferative skin disease often complicated by bacterial infections (12, 23). The mortality can be as high as 30 % and recovering animals may develop lifelong immunity (12). With no therapy at hand, prophylactic vaccination seems to be a promising approach to protect camels. Higgins (8) reported about early attempts of nomads to protect their animals by exposing them to scabs from recent outbreaks ground in milk. In 1992, calves were vaccinated with vacciniavirus (Orthopoxvirus commune) (8), and in the same year homologous vaccines - derived from CPV isolates serially passaged in cell culture - were described by two groups (7, 10). According to both studies homologous vaccination proved to be effective after subsequent challenge in a limited number of animals. However, no data are available that would allow discrimination of the vaccine strains from wildtype isolates.

In this study data on biological characteristics of two serially passaged CPV isolates and their progenies are presented. In a mouse model, passageation is shown to have led to a significant decrease in virulence.

MATERIALS AND METHODS

Viruses and cells
CPV strains variole de dromadaire 49 (VD49) and variole de dromadaire de Maurétanie (VDM) have been isolated from camels with generalized skin lesions in Niger (VD49) and Mauretania (VDM) (kindly provided by Dr. N. Ba-Vy, CIRAD-EMVT). Both strains were subcultured five times on MA-104 cells (African green monkey kidney cell line). In the following, these 5th passages will be referred to as wildtype virus (VDM wt and VD49 wt, respectively). After 107 passages on Vero cells the strains were plaque purified three times. Subsequently, the 114th passages (referred to as VDM 114 and VD49 114, respectively) were investigated.

Replication was assessed in the following cell lines: BHK 21 (baby hamster kidney), Dubca (camel skin; kindly provided by Prof. Dr. O.-R. Kaaden, Munich), E.Derm (equine dermal fibroblasts), L929 (murine subcutis), MA-104 (African green monkey kidney), MDBK (Madin Darby bovine kidney), MDCK (Madin Darby canine kidney) and Vero (African green monkey kidney). For microscopic inspection, cell lines were subcultured on cover slips in Leighton tubes, and infected with freeze-thawed cell culture material of VDM and VD49 wt, respectively. After 1 h at 37°C, cells were washed once and culture continued. Cells were fixed at 6, 12, 24, 30, 36, 42, 48, 72 and 120 h post infectionem (p.i.) with ethanol-glacial acetic acid (3:1), stained with hematoxylin-eosin and embedded (16). In order to compare
Animal experiments

Suckling F81 POP mice (27) were infected with 10-fold dilutions (10^9 to 10^-6) of purified virions of VDM and VD49 wt and 114, respectively, 36 ± 8 h after parturition. Six animals received 0.1 ml intraperitoneally (i.p.) and 7 to 8 animals received 0.02 ml intracerebrally (i.cer.). The titer of each virus preparation was determined on Vero cells (11). Untreated animals or animals inoculated with dilution buffer (2.5 mM Tris - 1 mM EDTA, pH 8.0) served as controls. Infected animals were inspected twice a day. Animal experiments were approved by the government of Oberbayern (reference nbr. 211-2531-28/93).

Restriction enzyme analysis

Viral DNA of both CPV strains was isolated from purified virions from passages 0 (wildtype), 30, 80 or 82, respectively, 36 ± 8 h of incubation. DNA was cleaved with restriction endonucleases HindIII and XhoI according to manufacturer's instructions (Boehringer, Mannheim, Germany). Resulting fragments were analyzed in 0.4 % and 1.2 % agarose gels and their molecular weights were estimated by comparison with molecular weight standards (1 KB ladder and HMW marker; Pharmacia, Freiburg, Germany). The total size of each genome was determined by summation. Southern blots were prepared on Hybond N+ membranes (Amersham/Buchler, Braunschweig, Germany) using 0.4 N NaOH as transfer buffer. For hybridization restriction enzyme generated DNA-fragments of CPV, vaccinia virus strain Elstree and cowpox virus strain Brighton were gel-isolated and labeled according to manufacturer's instructions (DIG-DNA-Labeling and Detection Kit Nonradioactive; Boehringer, Mannheim, Germany).

RESULTS

Replication of VDM and VD49 in vitro

Replication of VDM and VD49 wt and 114, respectively, was observed in cell lines derived from various hosts. Generally, 12 to 24 h.p.i. rounding of cells could be seen. The formation of visible plaques became evident after 24 h.p.i. On the second day plaque diameters averaged from 0.2 mm to 0.5 mm on most cell lines and up to 1.5 mm on Dubca cells. A characteristic feature of CPV is the formation of multinucleated giant cells. This was most distinct in African green monkey kidney cell lines Vero and MA-104 with 20 to 200 nuclei involved. Some differences in virus yield and plaque size were observed between wt and passage 114. The formation of comets was only seen with the highly passaged strains. Most obvious was the failure of both virus strains from passage 114 to replicate in MDCK cells and in Dubca cells. Forty-eight hours after infection the titer of wildtype viruses had increased at least 400-fold (VDM wt) and 6000-fold (VD49 wt), respectively, whereas no increase was observed for VDM 114 and VD49 114 infected cells as demonstrated for Dubca cells in figure 1. Furthermore, agglomerations of rounded cells was observed in BHK, E.derm (except VDM) and L929 cells after infection with wildtype strains and only in E.derm cells after infection with passages 114. Table I summarizes the differences in cytopathic effects of both strains in different cell lines (rounding up of cells, plaque formation and plaque diameter up to 0.5 mm are not noted).

Figure 1: replication of camelpoxvirus (CPV) strains VDM and VD49 in Dubca cells. The values represent the titers (plaque forming units (pfu)) after 1, 24, 48, 72 and 120 h post infectionem (determined on MA104 cells).
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TABLE I

Differences in cytopathic effects (cpe) observed in various cell lines after infection with camelpoxvirus (CPV) strains VDM and VD49 wt (wildtype) and 114 (passage 114), respectively

| Cell line | VDM 114 | VDM wt | VD49 114 | VD49 wt |
|-----------|---------|--------|----------|---------|
| BHK       | AG(20–50)| -      | AG(10–50)| -       |
| Dubca     | GC(Dia(1.3)| -      | GC(Dia(1.0)| -       |
| L29       | AG(20–20)| -      | AG(5–10)| -       |
| WA-104    | GC(50–200)| -      | GC(50–100)| -       |
| MDCK      | GC(50–200)| -      | GC(50–100)| -       |
| Vero      | GC(50–10)| GC(15) | GC(20–50)| GC(20–15) |

cpe, like rounding up of cells, plaque formation and plaque diameters up to 0.5 mm are not particularly noted.
AG = agglomeration (in brackets: number of cells involved); CF = comet formation; GC = giant cell (in parentheses: number of nuclei involved); Dia = diameter (in parentheses: diameter in mm); -0 = no effect was seen, neither rounding up nor plaque formation.

Figure 2: mortality of suckling mice after intraperitoneal (i.p.) or intracerebral (i.cer.) infection with camelpoxvirus (CPV) strains VD49 wt and VD49 114.

Figure 3: mortality of suckling mice after intraperitoneal (i.p.) or intracerebral (i.cer.) infection with camelpoxvirus (CPV) strains VDM wt and VDM 114.

A marked attenuation was seen after application of the highly passaged viruses VDM 114 and VD49 114. The LD50 p.m. was calculated to be in the range of 1 to 7 x 10^6 pfu for both strains and both modes of application (figure 2, 3 and table II).

Genome analysis

The restriction fragments obtained after digestion of different passages of VDM and VD49 with either XhoI or HindIII are shown in figure 5. The majority of fragments were comigrating. However, some fragments present in the wt strain (i.e. 11, 14.5 and 18 kilobasepair (kbp) HindIII-fragment of VDM) were absent in subsequent passages. In contrast, some new fragments could be
Comparison of LD$_{50}$ values for one mouse after application of camelpoxvirus strains VDM and VD49 wt (wildtype) and 114 (passage 114), respectively

| Virus passage | Virus titer [pfu]$^1$ | Application$^2$ | LD$_{50}$ p.m. [pfu]$^3$ | Increase in LD$_{50}$$^4$ |
|---------------|----------------------|-----------------|-------------------------|-------------------------|
| VDM wt        | 9.0 x 10^5           | i.p.            | 3                       | 260,000                 |
| VDM 114       | 8.4 x 10^5           | i.p.            | 7.8 x 10^5              |                         |
| VDM wt        | 1.8 x 10^5           | i.cer.          | 2.5                     |                         |
| VDM 114       | 1.7 x 10^5           | i.cer.          | 2.1 x 10^5              |                         |
| VD49 wt       | 1.5 x 10^5           | i.p.            | 5                       |                         |
| VD49 114      | 4.1 x 10^5           | i.p.            | 3.3 x 10^5              |                         |
| VD49 wt       | 3.0 x 10^5           | i.cer.          | 225                     |                         |
| VD49 114      | 0.8 x 10^5           | i.cer.          | 1.3 x 10^5              |                         |

1 amount of plaque-forming units (pfu) in the applied volume (0.1 ml i.p.; 0.02 ml i.cer.) of stock virus (10$^9$) as determined on MA-104 cells
2 kind of application: i.p. (intraperitoneal) or i.cer. (intracerebral)
3 LD$_{50}$ p.m. (p.m.) in pfu (based on virus titer and mortality rate, figures 2 and 3)
4 increase of the LD$_{50}$ in the ratio of passage 114 to wt

Detected (i.e. 7.9 kbp fragment of VDM and 10.1 kbp fragment of VD49). The total size of each genome was obtained by summing up. One hundred fourteen passages did not result in any changes of the length of VD49 as compared to the wt strain (about 166.5 kbp). However, the size of the genome of VDM 114 had decreased by about 22 kbp (172 kbp versus 194 kbp) compared to the wt virus. Physical maps were established for wildtype and passage 114 of both strains (figure 6) and the difference could be attributed to deletions in the right and left termini (reducing their size 17.5 and 4.5 kbp, respectively).

**DISCUSSION**

The pheno- and genotype of two CPV isolates (VDM and VD49) were examined before and after serial in vitro passages. Both wildtype viruses displayed similar characteristics as compared to data previously described (1, 2, 4, 13, 14, 23, 24, 26). However, after passagation differences were noted in vivo and in vitro. It seems remarkable that both strains have lost the ability to replicate in Dubca cells that originated from camel skin cells. The second difference noted is the formation of comets that could be seen with the passaged viruses in MA-104 cells. Formation of comets is caused by a unidirectionally spread of extracellular enveloped virions (EEV). It is well accepted that EEV plays an important role in the pathogenesis in vivo and might contribute to a protective immune response (25). This is of course essential for an effective vaccine.

However, the essential part of this study was the comparison of virulence of VDM and VD49 before and after cell culture passagation in a mouse model. According to data described in references 2, 4, 13, 14, 26, the susceptibility of mice seems to be age dependent. In this study, 36 ± 8 h old mice proved to be highly susceptible for both VDM wt and VD49 wt. After intracerebral application, the pathogenicity of VD49 wt was slightly lower than the pathogenicity of VDM wt. Comparing the modes of application, intraperitoneal inoculation leads to a higher mortality and results can be obtained within six days, which should be of help in future experiments.

However, a striking low pathogenicity of cell culture passaged virus could be demonstrated. Comparing the LD$_{50}$ p.m. values, the amount of pfu increased up to 260,000-fold. Only a 10,000-fold increase was described for recombinant vaccinaviuses with a thymidin kinase negative phenotype (3). It can only be assumed that during passagation mutations and/or deletions have occurred. It is tempting to speculate that failure to replicate in Dubca cells (the reasons are unknown) point to a highly attenuated phenotype in camels too.

Of interest are two animals which received a dilution (10$^{-3}$ and 10$^{-5}$ fold) of VDM 114 i.p. and were eaten by their dams. Therefore, an examination was not possible. However, in both cases only one animal from a group of seven was missing and because no animal of the other groups died (10$^{-1}$, 10$^{-2}$ and 10$^{-4}$ fold virus dilutions) these two animals were not included in the survey on the LD$_{50}$ value.

Investigation of the viral genome of VDM and VD49 indicates two different ways of genomic evolution during cell culture passages: VDM wt displays migration patterns and an overall genomic size similar to other CPV strains (5, 17, 24). Continuous passagation leads to a loss of 22 kbp in the terminal regions and thereby probably to a loss of genes which are not essential for replication in vitro. Many authors (6, 9, 15, 22) assume that in the variable-sized termini proteins are encoded which interfere with the host’s immune response.

In contrast, the mouse pathogenic VD49 wt displays a significantly shorter genome than VDM wt and other CPV strains...
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118 (165 versus 190 kbp) (5, 17, 24) due to a truncated right terminus, thus resembling the VDM 114 strain with a highly attenuated phenotype. Passagation of VD49 wt leads only to a loss of approximately 1 kbp but is associated with a marked attenuation.

The mechanisms leading to attenuation by cell culture passagation are not yet understood. Maybe minute deletions or even point mutations in genes interfering with the host's immune response are responsible for the loss of pathogenicity (15, 22). Thus, further detailed investigations like marker rescue experiments would be helpful to identify the gene(s) involved in virulence. However, at present molecular investigations cannot replace animal experiments for the proof of apathogenicity for animals or man.

The results show that VDM 114 and VD49 114 might be candidates for live vaccines against CPV infection. However, this can only be evaluated in the natural host and must include subsequent challenge experiments. Thereby, two characteristics (failure to replicate in Dubea cells and formation of comets) could serve as important in vitro markers to distinguish the attenuated strains from field strains.

Figure 5: electropherogram of Hind III or XhoI digested DNA of camel poxvirus (CPV) strains VDM and VD49 obtained from different passages. Arrows mark the terminal fragments.

S = molecular weight standard

1 = VDM wt
2 = VDM passage 30
3 = VDM passage 80
4 = VDM passage 110
5 = VDM passage 114
6 = VD49 wt
7 = VD49 passage 30
8 = VD49 passage 82
9 = VD49 passage 110
10 = VD49 passage 114
Acknowledgements

The authors are grateful to Dr. Nguyen Ba-Vy, CIRAD-EMVT, Maisons-Alfort, France, for kindly providing virus strains VDM and VD49 and Professor O.-R. Kaaden, Institute of medical microbiology, infections and epidemic diseases, Munich, Germany, for kindly providing the cell line Dubca. The authors also thank Mrs. Laura Chaudhuri and Mrs. Gudrun Zoller for excellent technical help.

REFERENCES

1. BA-VY N., RICHARD D., GILLET J.P., 1989. Propriétés d'une souche d'orthopoxvirus isolée des dromadaires du Niger. Revue Elev. Méd. vét. Pays trop., 42 (1): 19-25.
2. BAXBY D., 1972. Smallpox-like viruses from camels in Iran. Lancet, 2: 1063-1065.
3. BULLER R.M.L., SMITH G.L., CREMER K., NOTKINS A.L., MOSS B., 1985. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidin kinase negative phenotype. Nature, 317: 813-815.
4. DAVIES F.G., MUNGAI J.N., SHAW T., 1975. Characteristics of an Kenyan camelpoxvirus. J. Hyg. Camb., 75: 381-385.
5. ESPOSITO J.J., KNIGHT J.C., 1985. Orthopoxvirus DNA: A comparison of restriction profiles and maps. Virology, 143: 230-251.
6. GOEBEL S.J., JOHNSON G.P., PERKUS M.E., DAVIES S.W., WINSLOW J.P., PAOLETTI E., 1990. The complete DNA sequence of vaccinia virus. Virology, 179: 247-266.
7. HAFEZ S.M., AL-SUKAYKAN A., DELA CRUZ D., MAZLOUM K.S., AL-BORKMY A.M., AL-MUKAYEL A., AMJAD A.M., 1992. Development of a live cell culture camelpox vaccine. Vaccine, 10: 533-539.
8. HIGGINS A.J., SILVEY R.E., ABDELGHAIFIR A.E., KITCHING R.P., 1992. The epidemiology and control of an outbreak of camelpox in Bahrain. In: Proceedings of the 1st International Camel Conference, Dubayy, United Arab Emirates, 2-6 February 1992. New Market, United Kingdom, R&W Publications, p. 101-104.
9. JOHNSON G.P., GOEBEL S.J., PAOLETTI E., 1993. An update on the vaccinia virus genome. Virology, 196: 381-401.
10. KAADEN O.-R., WALZ A., CZERNY C.-P., WERNERY U., 1992. Progress in the development of a camelpox vaccine. In: Proceedings of the 1st International Camel Conference, Dubayy, United Arab Emirates, 2-6 February 1992. New Market, United Kingdom, R&W Publications.
11. KAEBER G., 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenuntersuchungen. Arch. exp. Pathol. Pharmacol., 162: 480.
12. KRIZ B., 1982. A study of camelpox in Somalia. J. comp. Path., 92: 1-8.
13. MAHANIEL H., BARTENBACH G., 1973. Systematisierung des Kamelpockenvirus. Zbl. Vet. Med. B, 20: 572-576.
14. MARENNIKOVA S.S., SHENKMAN L.S., SHELUKHINA E.M., MALT SEVA N.N., 1974. Isolation of camelpox virus and investigation of its properties. Acta virul., 18: 423-428.
15. MASSUNG R.F., ESPOSITO J.J., LIU L., QI J., UTTERBACK T.R., KNIGHT J.C., AUBIN L., YURAN T.E., PARSONS J.M., LOPAREV V.N., SELIVANOV N.A., CAVALLARO K.F., KERLAVAGE A.R., MAHY B.W.J., VENTER J.C., 1993. Potential virulence determinants in terminal regions of variola smallpox virus genome. Nature, 366: 740-751.
Characterisation of two camelpoxvirus isolates

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Caractérisation in vitro et in vivo de deux souches de virus de la variole des dromadaires à virulence atténuée

Deux souches de virus de la variole des dromadaires (CPV) isolées de dromadaires affectés d'altérations cutanées généralisées ont été soumises à des passages en série de cellules Vero. Différentes caractéristiques phénotypiques furent étudiées in vivo et in vitro et comparées à celles des souches sauvages correspondantes. A beaucoup d'égards, aucune différence ne put être constatée. Toutefois, dans un modèle de souris, les deux souches soumises au passage s'avèrent sensiblement atténuées. De plus, ces souches ne sont pas en mesure de se reproduire dans une culture dérivée de cellules cutanées de dromadaire. Par comparaison des cartes génomiques physiques établies pour les enzymes HindIII et Xhol, des délétions totalisant 22 kilobases furent constatées pour l'une des souches atténuées. Le génome de la deuxième souche ne comportait que des altérations mineures.

Mots-clés : Dromadaire - Camelus dromedarius - Camelus bactrianus - Orthopoxvirus - Expérimentation in vitro - Expérimentation in vivo - Vaccin.

Résumé

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Caractérisation in vivo e in vitro de dois aislamientos de Camelpoxvirus con virulencia disminuida

Se aislaron dos cepas de Camelpoxvirus (CPV) en camellos con enfermedad cutanea generalizada, las cuales fueron luego sometidas a pasages en serie en células Vero. Se investigaron varias propiedades fenotípicas in vitro e in vivo, en comparación con aquellas correspondientes a las cepas de tipo salvaje. En muchos aspectos no se observaron diferencias. Sin embargo, en un modelo de ratones, ambos pasages de las cepas demostraron ser altamente atenuantes. Además de esto, ninguna de las cepas logró replicarse en una línea celular derivada de células de piel de camello. La comparación de los mapas físicos establecidos para las enzimas HindIII y Xhol revelaron errores de un total de 22 kbp en una cepa atenuada. En la segunda cepa se notaron solamente alteraciones menores.

Palabras clave : Dromedario - Camelus dromedarius - Camelus bactrianus - Orthopoxvirus - Experimentación in vitro - Experimentación in vivo - Vacuna.