Arch Virol (1997) 142: 807–821

Archives of Virology

Identification of three distinct antigenic sites in parapoxviruses

C.-P. Czerny[1,2], **R. Waldmann**[1], and **T. Scheubeck**[1]

[1] Institute for Medical Microbiology, Infectious and Epidemic Diseases,
[2] WHO Collaborating Centre for Collection and Evaluation of Data on Comparative Virology, Veterinary Faculty, Ludwig-Maximilians-University, Munich, Federal Republic of Germany

Accepted September 4, 1996

Summary. Monoclonal antibodies (Mabs) were generated in BALB/c mice immunized with gradient-purified particles, envelopes and cores of intracellular mature orf virus D-1701. Three distinct antigenic sites were identified in this virus strain. Their topographical relationships was determined by pairwise epitope specificity studies in competition ELISAs. One MAb (class IgM) neutralized virus infectivity. Four μg/ml purified IgM gave a 50% reduction of 100 PFU of orf virus D-1701. As shown by immunogold electron microscopy (ELMI), all MAbs reacted with epitopes localized on the virus surface. Western blotting analysis demonstrated that two proteins of a Mr of 39 kDa and 22 kDa were the main targets for the Mabs. Cross-reactivity studies of several parapoxviruses (PPV) differentiated stomatitis papulosa virus strains from orf virus and milker's node virus (MNV) by a missing antigenic site.

Introduction

The genus Parapoxvirus (PPV), a member of the Poxvirus family, represents a group of viruses closely related to their morphological, biological and serological relationships [4, 23, 46]. The prototype-species is orf virus which infects sheep and goats causing a contagious pustular dermatitis. It can also be transmitted to humans by adventitious contact [13, 35]. Two further members are PPV bovis 1, the causative agent of bovine papular stomatitis (BPS) and PPV bovis 2, the pseudocowpox or milker's node virus (MNV) in man. Additionally, a poorly characterized group of PPV is responsible for localized or generalized cutaneous lesions in camels, wildlife ruminants or seals [8, 10, 17, 19, 26, 31, 32, 39, 45].

Poxviruses are able to maintain and express large amounts of foreign DNA. Therefore, orf virus is also under intensive scrutiny as a potential immunization vector of human and veterinary importance [25, 36]. The interest so far focused on highly attenuated and genetically stable viruses [12].

Parapoxviruses replicate in the cytoplasm of infected cells. Morphogenesis results in two distinct infectious forms of virus particles [30, 38]. It seems likely that they correspond to the two types detected in orthopoxviruses. The majority of virus particles are intracellular mature virus (IMV) with an electron-dense core surrounded by a single envelope. A small proportion of extracellular enveloped virus (EEV) is enwrapped in an additional Golgi-derived envelope.

About 30–40 structural proteins have been detected in IMV and EEV [4, 28, 38, 42]. Monoclonal antibodies were established recognizing orf virus directed cell surface antigens [23]. However, in contrast to orthopoxviruses (OPV) little is presently known about the functions of orf virus polypeptides in early virus/host interactions or in the induction of specific and protective B- and T-cell mediated immune responses in animals. Parapoxviruses do not guarantee long lasting immunity in animals [35]. In humans, however, immunity to orf virus lasts lifelong according to several reports (for references see [47]. There is strong evidence that a polypeptide of about 40 kilodaltons (37 kDa-45 kDa) [1, 4, 28, 42, 47] is the major component of the surface tubules. It seems to be responsible for induction of neutralizing antibodies in the host. From the data of DNA sequence analysis, homologues of genes for the vaccinia virus (VV) 14 kDa fusion protein [37] encoded by the VV Copenhagen open reading frame (ORF) A27L [14] and for the vaccinia virus 35 kDa envelope protein (H5R) [15] were found in orf virus [11, 34]. However, the corresponding proteins were not identified. Recently, another open reading frame (BamHI B2L) in orf virus New Zealand 2 (NZ2) was mapped encoding a 42 kDa protein. When the protein was expressed in VV and delivered to sheep, it induced strong immune responses [41]. This protein corresponds to the major envelope antigen (p37K) of vaccinia virus EEV particles [3, 18] as well as to the p43 kDa proteins of fowlpox [5] and molluscum contagiosum virus [2].

Detailed knowledge about PPV structural proteins is desirable to characterize immunodominant antigenic sites and to understand the molecular basis of virus-host interactions. In this communication we report about the establishment of neutralizing and non-neutralizing MAbs against orf virus D-1701. This enabled us to identify and characterize three distinct antigenic sites in IMV particles and subviral preparations of orf virus. Additionally, we calculated antigenic relationships between several PPV strains.

Materials and methods

Cells and viruses

Primary embryonic lung cells cultured in minimum essential medium (MEM) and supplemented with 5% fetal calf serum were used to propagate the highly attenuated orf virus vaccine strain D-1701 [27], orf viruses B047 and S1 Japan, stomatitis papulosa virus strains (PPV bovis-1) BPS B-177, BPS Iran, BPS V660, and the milker's node virus (PPV bovis-2) B075. Additionally, orf virus D-1707 was adapted to the permanent monkey kidney cell line MA-104. Infectivity titres were determined on 24-well Linbro plates (Nunc, Wiesbaden) and calculated as plaque forming units (PFU). All virus preparations were purified and concentrated by sucrose gradient centrifugation [6, 20]. The purified preparations consisted of

IMV. Protein contents of the samples were determined by the method of Lowry and co-workers [24].

Controlled degradation of orf virus into envelopes and cores

The preparation was according to the procedure of Easterbrook [9] with a slight modification [42]. Purified orf virus D-1701 (2 000 μg/ml) was resuspended in phosphate-buffered saline (PBS), dialysed and incubated with 1% NP-40 and 50 μl 2-mercaptoethanol (ME) for 60 min at 37 °C. The mixture was sonicated for 10 sec and centrifuged through a 3.5 ml sucrose cushion (36% wt/vol) in a Beckman rotor SW 60 Ti at 40 000 rpm (230 000xG) and 4 °C for 45 min. The pellet (cores) was resuspended in PBS, the supernatant above the cushion (envelopes) was dialysed against PBS.

Preparation of polyclonal hyperimmune sera

Polyclonal hyperimmune sera against purified orf virus D-1707, core, and envelope preparations were raised in rabbits (Chinchilla Bastard) by four subcutaneous injections of 175 μg antigen/animal at intervals of three weeks.

In the same way a 2-year-old female Merino sheep was immunized with the purified orf virus D-1701.

Sera of immunized mice used as spleen donors for monoclonal antibody production were also harvested.

Monoclonal hybridoma technique

Monoclonal antibodies against orf virus D-1701 were produced in BALB/c-mice immunized intraperitoneally with 30 μg of the purified, live virus strain or with the same amounts of envelope and core preparations. Three booster injections followed at intervals of 14 days. BALB/c-spleen cells were harvested and fused with the mouse myeloma cell line P33-X63Ag8.653 [21]. Supernatants of growing hybridoma colonies were screened for antibody production. Specificity of the MAbs was determined by indirect ELISAs on microplates (Immuno II, Nunc, Wiesbaden) coated with 1 μg/ml of the purified orf virus D-1701, and by a plaque-reduction-test (PlRT) using 100 PFU of the same virus strain and 5×10^5 MA-104 cells/ml [6]. The Elstree strain of vaccinia virus served as the negative control antigen in both assays. Hybridomas secreting specific antibodies were cloned twice. The cells were propagated in culture. Immunoglobulin (Ig) classes and subclasses of serum-free hybridoma supernatants were determined by ELISA with anti-mouse isotype-specific antisera (Sigma, Munich). IgG was isolated from tissue culture fluids by affinity chromatography on protein G-Sepharose columns. IgM was purified by ConA-Sepharose columns (Pharmacia, Freiburg). Purified MAbs were conjugated with Biotin-X-NHS dissolved in dimethylformamide (Sigma, Munich) by standard methods (16).

Competition ELISA

Purified MAbs were adjusted to a protein concentration of 100 μg/ml in PBS and titrated in twofold steps on microplates coated with 1 μg/ml of orf virus D-1701. Subsequently, the MAb-biotin-conjugates were added in the optimal working dilutions, giving a defined photometer extinction (OD_{450nm}), which ensured sufficient saturation of all free epitopes. Plates were incubated for 60 min at 37 °C before horseradish-peroxidase (HRP)-conjugated avidin (Sigma, Munich), 3, 3′, 5, 5′-tetramethylbenzidine (TMB), and 2M H_2SO_4 were added as detecting reagents (6). Between each step microplates were washed five times. Reduction of

the photometer extinction of the conjugated MAbs by the competing MAbs was calculated by the formula %-inhibition = [1 − OD_{450nm} (MAb + MAb-conjugate)/OD_{450nm} MAb-conjugate] × 100. The binding curves were plotted. A reduction of the photometer extinction ≥ 50% was regarded as a significant inhibition.

Electron microscopy and immunogold labeling technique

Purified orf virus D-1701 (100 µg/ml) was adsorbed for 1 min to Formvar-coated and carbon-vaporized 300-mesh grids, contrasted for 15 sec with phosphoric tungstic acid, dried, and examined in a transmission electron microscope (Zeiss EM 10 C/R). Between each step grids were washed thoroughly with Tris-buffered saline (TBS).

Serum-free MAb-containing cell culture supernatants and anti-mouse IgG-gold conjugate (particle size 10 nm; Sigma, Munich) were taken for the immunogold labeling technique [6, 33].

SDS-PAGE, isoelectric focussing, and western blotting

Proteins of purified PPV strains (10 µg/slot) were fractionated on vertical 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels [22]. The proteins were silver-stained [29] or subsequently transferred to nitrocellulose membranes [43]. Immunodetection was performed by standard techniques using monoclonal tissue culture fluids or immune sera (1:5 or 1:150), HRP-conjugated anti-IgG antisera (1:250), and HRP colour developing reagent (Bio-Rad, Munich). The Mr of stained viral proteins was determined in a Beckman gel mate 1000 sonic digitizing system (Beckman, Munich) on the basis of Mr standards from 205 kDa to14.3 kDa (Sigma, Munich).

For two-dimensional electrophoresis the IPG-Dalt-technique was used. In the first dimension proteins were separated by isoelectric focussing (IEF) on 0.5 mm thick IPG-gels with immobilized PI-gradients (4.0–10.0; Pharmacia, Freiburg) and subsequently transferred to SDS-PAGE which followed as the second dimension. Proteins were detected by silver staining or Western blotting.

Results

Pairwise epitope specificity studies by competition ELISAs

For MAb production, BALB/c mice were immunized with total virus, envelopes, and core preparations of orf virus D-1701. After various fusion experiments, four stable hybridoma colonies could be obtained secreting specific anti-orf virus MAbs. Three MAbs (anti-orf-4D9/2G7, IgM; anti-orf-3C5/2G3, IgG3; anti-orf-8G7/1G3, IgG3) resulted from different mice after immunization with purified orf virus D-1701 and one MAb (anti-orf-7F7/2F6, IgG1) was achieved after immunization with an envelope preparation. No MAbs derived from mice immunized with core particles. In order to determine the number of distinct orf virus epitopes, all MAbs were purified and tested pairwise against each other in competition ELISAs. Two MAbs were considered to recognize separate epitopes if they bound independently of each other to the antigen, and no inhibition occurred. Inhibitions of 100% and inhibition curves identical in their kinetics indicated that two MAbs recognized the same epitope.

The analysis demonstrated that three different orf virus epitopes were detected by the four cross-reacting MAbs (Table 1). However, the antigenic sites

Table 1. Pairwise epitope specificity studies by competition ELISAs[a]

Competing antibodies	Conjugated antibodies			
	MAb α[b]-orf 3C5	MAb α-orf 4D9	MAb α-orf 8G7	MAb α-orf 7F7
MAb α-orf 3C5	**100**	--[c]	100	82
MAb α-orf 4D9	90	**100**	89	94
MAb α-orf 8G7	100	--	**100**	**77**
MAb α-orf 7F7	66	--	64	**100**

[a] Competition in binding to their antigenic sites was tested with purified (100 μg/ml)and biotin-conjugated MAbs in a pairwise competition ELISA. Microplates were coated with 1 μg/ml of orf virus D-1701. A reduction of the photometer extinction (OD_{450nm}) $\geq 50\%$ indicated two MAbs binding to identical or closely related antigenic sites

[b] α = anti

[c] Blocking $< 5\%$

were regarded as closely related because the MAbs interfered with each other's binding to different degrees ($\geq 50\%$, but $\leq 100\%$). The MAb anti-orf-4D9 was able to block all other MAbs with values of 89–94% but reversely it was not inhibited. The epitope was designated as epitope 1. The other three MAbs could influence each other reciprocally when binding to their specific sites. The MAb anti-orf-7F7 recognized epitope 2 and was blocked by all other MAbs (77–94%) vice versa. However, it was only able to block the MAbs anti-orf-3C5 and anti-orf-8G7 (64–66%). These two MAbs had identical inhibition curves. Therefore, it was postulated that they recognized the same epitope 3. Both MAbs could be inhibited by the MAbs anti-orf-7F7 and anti-orf-4D9 (64–90%).

An identical test was made using a rabbit and sheep hyperimmune serum against orf virus D-1701. Both sera inhibited all MAbs (81–100%). The MAbs were able to block the sheep serum (58–91%), but only the MAb anti-orf-4D9 competed with the rabbit serum (data not shown).

Identification of neutralizing epitopes

Neutralizing epitopes were detected by PRT. Purified MAbs were adjusted to 10 μg/ml and titrated against 100 PFU of orf virus D-1701. Only MAb anti-orf-4D9 (epitope 1) had neutralizing capacity. The IgM concentration of 4 μg/ml led to a 50% plaque reduction of the test virus.

Analogous experiments were executed with hyperimmune sera obtained from rabbits immunized with total virus, envelopes or cores of orf virus D-1701. Neutralizing antibody titres in the PRT were 1:800, 1:100 or 1:8, respectively. The sheep hyperimmune serum neutralized orf virus up to a titer of 1:16 000.

Immunogold electron microscopy

Specific binding of the MAbs to the virus surface could be demonstrated by electron microscopy. Grids coated with purified orf virus D-1701 were incubated with the MAbs and then contrasted with gold-conjugated anti-mouse-IgG (particle size 10nm). One MAb against the vaccinia virus fusion protein (anti-VV-5B4, IgG2a) was used as a negative control [6, 7]. In the case of the MAb anti-orf-4D9 (epitope 1) the gold particles (fine dark dots) were bound evenly and very densely to the envelope of all the virus particles (Fig. 1A). The MAb anti-orf-7F7 (epitope 2) bound to the virus envelope (Fig. 1B), too. This site corresponded to that of the MAb anti-orf-4D9 but the distribution of the gold particles was somewhat sparser. The binding of the MAb anti-orf-3C6 (epitope 3) took place on a quantitatively smaller scale, too. The point of binding was considerably closer to the virus particle (Fig. 1C).

Identification of antigenic sites on orf virus proteins

The gradient-purified orf virus D-1701 as well as core and envelope preparations released by treatment of the virus particles with NP-40, 2-ME, and sonication, were fractionated on vertical 12% SDS-polyacrylamide gels under reducing conditions. Proteins were detected by silver staining. In purified orf virus about 30 bands of a Mr range of 14–226kDa could be identified with various degrees of resolution (data not shown). Polypeptides of 14kDa, 15kDa, 22kDa, 39kDa, 41kDa, and 66kDa were stained intensely. With the exception of the 39kDa band, these proteins could also be well detected in the core preparation. In the envelope fraction 12 proteins were stained in a Mr range of 15–77kDa. The 41kDa protein was absent, the 22kDa band was scarcely visible, and the 15kDa and 39kDa bands were considerably enriched.

For the detection of immunorelevant virus proteins, the gels were transferred to nitrocellulose membranes and incubated with immune sera or MAbs. A polyclonal rabbit hyperimmune serum against purified virus intensely stained the 66kDa, 39kDa, and 22kDa bands in total virus. Lighter staining was apparent to proteins of 56kDa, 25kDa, and 24kDa (Fig. 2). In cores the 39kDa protein was greatly reduced and hardly visible, the dominant bands were at 66kDa and 22kDa. In envelopes the 39kDa band was the major polypeptide, and also a 22kDa protein was stained quite well. Similar patterns were achieved with a sheep (Fig. 2) and a mouse hyperimmune serum (Fig. 3) against total virus. When using a rabbit hyperimmune serum against viral cores for immunodetection, the 66kDa and 22kDa proteins were well resolved in total virus and in cores. In these fractions the serum also detected a 35kDa and a 41kDa protein which were hardly visible in the envelope. The rabbit hyperimmune serum against envelopes demonstrated an enrichment of the 39kDa protein in total virus or envelopes. This was accompanied by a significant loss of the polypeptide in the core preparation. Serum from a non-immunized lamb had orf virus specific antibodies, too. They exclusively reacted with the 39kDa protein in total virus and in the envelope fraction. All the MAbs detected orfvirus antigens in Western

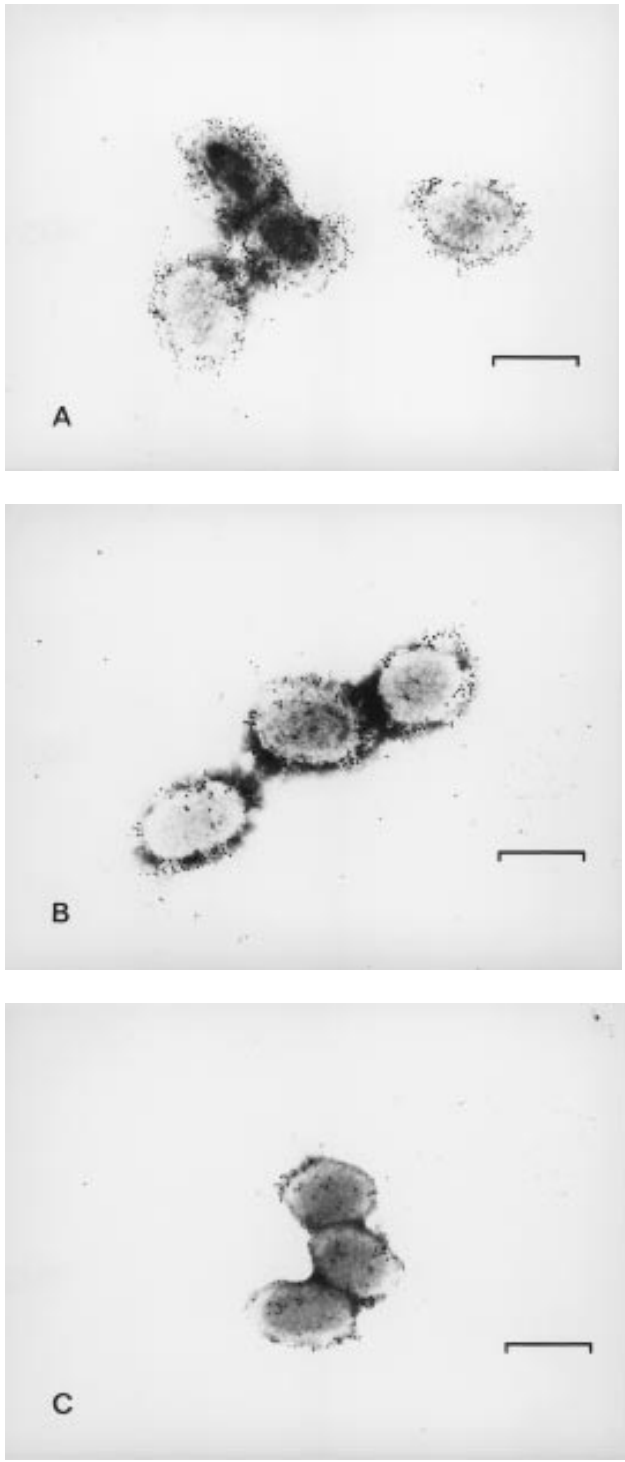

Fig. 1. Immunogold staining of surface proteins of highly purified orf virus D-1701. Specific attachment of MAb anti-orf- 4D9 (**A**), MAb anti-orf-7F7 (**B**), and MAb anti-orf-3C5 (**C**) is demomstrated at 80.000-fold magnification. Bars: 200 nm; gold particle size: 10 nm

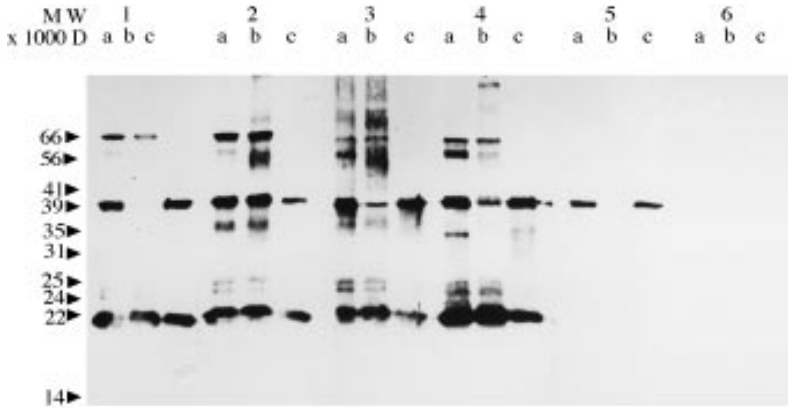

Fig. 2. Separation of gradient-purified orf virus D-1701 (*a*), cores (*b*), and envelopes (*c*) on 12% SDS-polyacryamide gel under reducing conditions. Detection of immunogenic proteins in Western blotting was performed by anti-orf rabbit-hyperimmune serum (*1*), anti-orf-core rabbit-hyperimmune serum (*2*), anti-orf-envelope rabbit-hyperimmune serum (*3*), anti-orf sheep-hyperimmune serum (*4*), pre-immune lamb serum (*5*), and anti-vaccinia rabbit-hyperimmune serum (*6*)

blotting (Fig. 3). The neutralizing MAb anti-orf-4D9 showed high affinity to the 39kDa protein in total virus or envelopes. It reacted very weakly with the core preparation. Faint bands of 56kDa, 31kDa, and 22kDa (total virus) were also detected by this MAb. The MAb anti-orf-7F7 obtained from a mouse imunized

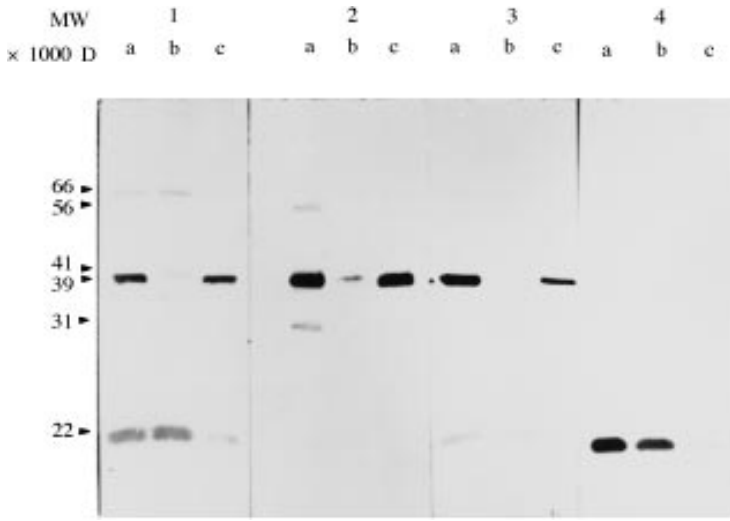

Fig. 3. Separation of gradient-purified orf virus D-1701 (*a*), cores (*b*), and envelopes (*c*) on 12% SDS-polyacryamide gel under reducing conditions. Detection of immunogenic proteins in Western blotting was performed by anti-orf mouse hyperimmune serum (*1*), MAb anti-orf-4D9 (*2*), MAb anti-orf-7F7 (*3*), and MAb anti-orf-3C5 (*4*)

by the envelope fraction, showed similar staining patterns with MAb anti-orf-4D9. The 39 kDa protein of total virus and envelopes showed a major reaction. The MAb anti-orf-3C6 dominantly bound to a 22 kDa protein. It was intensely stained in total virus and cores but to a very low extent in envelopes.

Distribution of the epitopes in different parapoxvirus strains

Binding of the MAbs to the three distinct antigenic sites in orf virus D-1701 was investigated by an indirect ELISA. Purified immunoglobulins were adjusted to 50 µg/ml and titrated in two-fold dilutions on microplates coated with 1 µg/ml of purified orf virus D-1701. The binding curves corresponded to Michaelis-Menten kinetics (Fig. 4). In the same way distribution of the three epitopes within eight orf and PPV-strains was tested by cross-reactivity of the MAbs. As demonstrated previously [6], we calculated Lineweaver-Burk-diagrams from the linear regression of each curve and determined the theoretical V_{max}(photometer extinction; $OD_{450\,nm}$) as well as the Michaelis-Menten constant (K_m). The latter value represents MAb concentration (ng/ml) at one-half V_{max} and provides information about MAb affinity [44]. Photometer extinctions

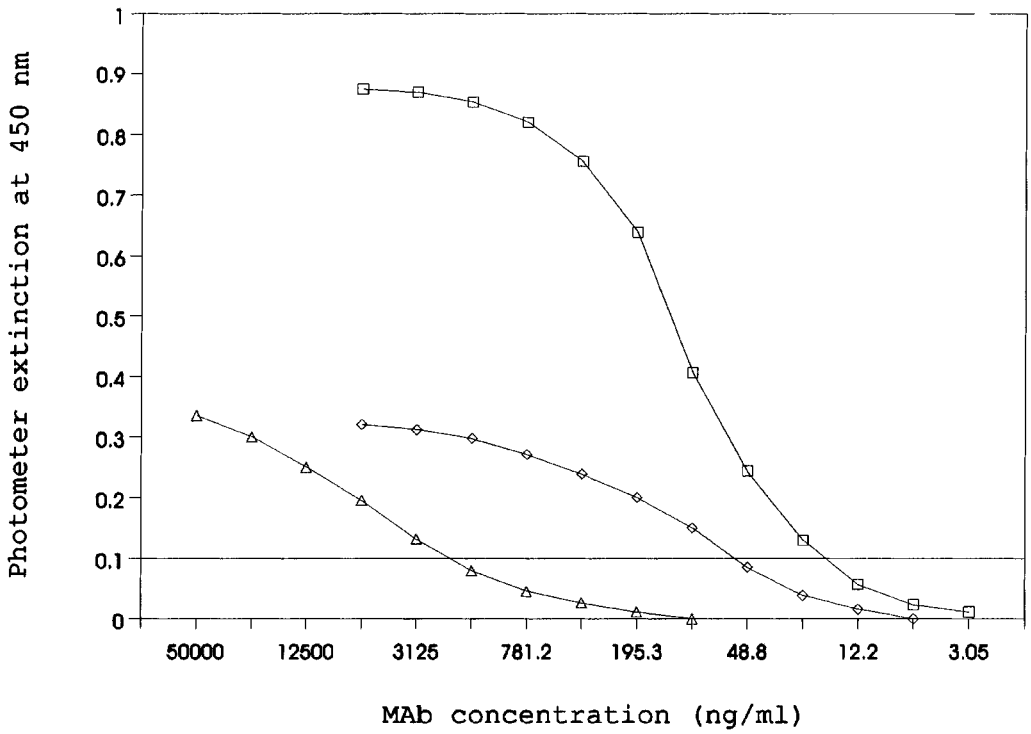

Fig. 4. Three distinct antigenic sites were detected in purified orf virus D-1701 by an indirect ELISA. Microplates were coated with 1 µg/ml purified antigen. The MAbs ◇ anti-orf-4D9 (epitope 1), △ anti-orf-7F7 (epitope 2), and □ anti-orf-3C5 (epitope 3) were adjusted to a concentration of 50 µg/ml and titrated in $\log_2$-dilutions. Photometer extinctions at 450 nm are shown

Table 2. Serological cross-reactivity of the MAbs with representative parapoxvirus strains in the ELISA[a]

Virus strains		Epitope 1 MAb 4D9	Epitope 2 MAb 7F7	Epitope 3 MAb 3C5
	Parapoxvirus Orf			
Orf virus D-1701 4.BEL	V^b_{max} =	0.349	0.359	0.942
	K^b_m =	121	5892	116
Orf virus D-1701 38.BEL	V_{max} =	0.331	0.371	0.982
	K_m =	137	5769	82
Orf virus B047	V_{max} =	0.344	0.401	0.980
	K_m =	122	5472	121
Orf virus S1- Japan	V_{max} =	0.324	0.389	0.962
	K_m =	115	5905	80
	Stomatitis papulosa virus (PPV bovis-1)			
Stomat. pap.[d] BPS B-177	V_{max} =	0.254	–[c]	0.818
	K_m =	179	–	105
Stomat. pap. BPS Iran	V_{max} =	0.275	–	0.823
	K_m =	130	–	120
Stomat. pap. BPS V660	V_{max} =	0.292	–	0.840
	K_m =	191	–	118
	Milker's node virus (PPV bovis-2)			
Milker's node MNV B075	V_{max} =	0.357	0.373	0.866
	K_m =	172	5713	100

[a] Epitope distribution in several PPV strains was investigagted by an indirect ELISA. Microplates were coated with 1 μg/ml purified orf virus D-1701. Purified MAbs (100 μg/ml) were titrated in $\log_2$-dilutions

[b] V_{max}-(maximal photometer extinctions at an OD_{450nm}) and K_m-values (IgG concentration in ng/ml at half-maximal binding) were calculated by linear regression analysis from MAb binding curves.

[c] The MAb anti-orf-7F7 missed its epitope in PPV bovis-1. Photometer extinctions were < 0.01

[d] Stomatitis papulosa virus

< 0.1. could not be included in the regression analysis, as these were too low and imprecise. They made it impossible to calculate the correct V_{max}. All data listed in Table 2 were calculated separately for each epitope from the V_{max} and K_m median values of identical antibodies. The epitopes 1 and 3 seemed to be genus specific

due to their presence in all parapoxvirus strains tested. No strain specific differences were apparent. The MAb anti-orf-4D9 recognized epitope 1 with V_{max}-values of 0.254–0.357 and K_m-values of 115–191. MAbs to the epitope 3 showed a higher affinity to V_{max} of 0.818–0.982 and K_m of 80–120. The antigenic site 2 was present in orf virus strains and in milker's node virus. It was not detected in pustular stomatitis virus strains. The corresponding MAb reached the same V_{max} (0.359–0.401) as the MAb anti-orf-4D9 against epitope 1, however, it had up to 50-times lower affinities (K_m = 5472–5904).

Discussion

In this communication we identified three distinct antigenic sites in highly purified intracellular mature orf virus particles. They were recognized by monoclonal antibodies and played also a role in induction of immune responses in several hosts such as sheep, rabbits, and mice. Epitope 1 was of great importance because it was responsible for virus neutralization. Together with epitope 2 it belonged to certain areas of the virus envelope which could clearly be shown by immunogold labeling, SDS-PAGE, and Western blotting. SDS-PAGE analysis allowed us to detect 33 proteins of purified orf virus D-1701 with Mr of 14–226 kDa. This figure agrees with the results of other authors [1, 4, 28, 42]. Thirty proteins were detected in the core preparation and 12 proteins in an envelope preparation. Only a few polypeptides were of immunological relevance. In total virus a 39/41 kDa double band was very prominent after silver staining. It was striking that this band was composed of a 41 kDa protein belonging to the viral cores and a 39 kDa band representing the major part of viral envelopes. After two-dimensional electrophoresis, with isoelectric focussing in the first and SDS-PAGE in the second dimension, the 39 kDa polypeptide consisted of four isomeres (pI 5.4–5.7; data not shown). In Western blotting analysis MAbs against epitopes 1 and 2 reacted intensely with this protein in preparations of total virus and envelopes. In the same way the band was stained by immune sera from rabbit, sheep, and mouse. The 41 kDa band was only recognized in viral cores by a rabbit serum exclusively produced against those subviral structures. Several investigations performed previously demonstrated that proteins in the Mr-region of 42–45 kDa, 37–kDa or 38.5 kDa were enriched in envelope preparations [1, 4, 42]. This was particularly observed when virus was disrupted according to the method of Stern and Dales [40]. This led to the conclusion that proteins within this range may be the major structural components of the surface tubules. McKeever and co-workers [28] reported of a 40 kDa envelope protein detected by all serum samples taken from 13 lambs short after an orf virus infection. Those antibodies were suspected to block spread of the virus in the body. In our study serum of a non-immunized lamb also detected the 39 kDa envelope component. Maternal antibodies, passively delivered via colostrum, were obviously responsible for this reaction. We could demonstrate by immunogold electron microscopy that the 39 kDa protein was localized in the virus envelope, however, there is no evidence that this protein is ka component of the

surface tubules. It played a significant role in virus neutralization since it was the main target for the neutralizing MAb anti-orf-4D9 (epitope 1). Analogously to our experiments with vaccinia virus [7], we were not able to isolate tubular structures by immune-affinity-chromatography with the MAb anti-orf-4D9 (data not shown).

In addition to the 39 kDa protein, a polypeptide of 22 kDa was stained to a high degree by all sera, with the exception of the pre-immune lamb serum. This protein consisted of two isomeres of pI 5.6 and 5.7 (data not shown) and was much more prominent in total virus and core particles than in the envelopes. Because this protein was recognized by the non-neutralizing MAb 3C5 and the corresponding epitope 3 unequivocally was localized on the virus surface after immunogold staining, it was difficult to relate this antigenic site to subviral structures. However, there was no doubt that the 22 kDa polypeptide was strongly associated to the core. Evidence for this phenomenon is given by data from Thomas and co-workers [42] describing the difficulty of milker's node virus to disrupt envelopes and cores. Very harsh procedures were required to completely separate both components. The close sterical correlation of the 39 kDa and 22 kDa polypeptides was also demonstrated by the fact that the corresponding MAbs showed a preferred and intensive reaction with one of these two bands in Western blotting. However, each MAb detected the other band to a very low level in total virus or subviral preparations. Pairwise epitope specificity studies performed by competition ELISAs emphasized this conclusion. The three distinct antigenic sites were closely related because the MAbs as well as hyperimmune sera of sheep and rabbit inhibited each other's binding to various degrees. To clarify these interactions, immunoscreening of a parapox/vaccinia genome library (kindly provided by A. Mercer) is currently performed and several recombinant vaccinia clones were already detected by the mono- or polyclonal antibodies (data not shown). This will enable the identification of the corresponding genes and allow a more precise epitope mapping. It may also contribute to a further differentiation of parapoxviruses. The immunological data are not sufficient because two of the immunodominant antigenic sites (epitopes 1 and 3) were well conserved within several parapoxvirus strains or isolates and seemed to be genus-specific. When antigen/antibody interactions were precisely calculated by linear regression of the MAbs' binding curves and Lineweaver-Burk plots were determined, K_m- and V_{max}-values were in a similar range in all virus strains tested so far. No significant strain-specific differences could be observed. Epitope 2, however, was homogenously distributed in orf and milker's node virus yet it was absent in PPV bovis-1 strains.

Acknowledgements

We are grateful to Andrea Jahnke for her excellent technical assistance and Ursula Wolf who assisted us with the electron microscopy and photographic documentation. We also thank Elke Bongers and Andrew Mercer for the critical review of the manuscript. This work was partly supported by the German Federal Ministry of Health.

References

1. Balassu TC, Robinson AJ (1987) Orf virus replication in bovine testis cells: kinetics of viral DNA, polypeptide, and infectious virus production and analysis of virion polypeptides. Arch Virol 97: 267–281
2. Blake NW, Porter CD, Archard LC (1991) Characterization of a molluscum contagiosum virus homologue of the vaccinia virus p37K major envelope antigen. J Virol 65: 3 583–3 589
3. Blasco R, Moss B (1992) Role of cell associated enveloped vaccinia virus in cell-to-cell spread. J Virol 66: 4 170–4 179
4. Buddle BM, Dellers RW, Schurig GG (1984) Heterogeneity of contagious ecthyma virus isolates. Am J Vet Res 45: 75–79
5. Calvert JG, Ogawa R, Yanagida N, Nazerian K (1992) Identification and functional analysis of the fowlpoxvirus homolog of the vaccinia virus p37K major envelope antigen gene. Virology 191: 783–792
6. Czerny C-P, Mahnel H (1990) Structrual and functional analysis of orthopoxvirus epitopes with neutralizing monoclonal antibodies. J Gen Virol 71: 2 341–2 352
7. Czerny C-P, Johann S, Hölzle L, Meyer H (1994) Epitope detection in the envelope of intracellular mature orthopoxviruses and identification of encoding genes. Virology 200: 764–777
8. Dashtseren T, Solovyev BV, Varejka F, Khokhoo A (1984) Camel contagious ecthyma (pustular dermatitis). Acta Virol 28: 122–127
9. Easterbrook KB (1966) Controlled degradation of vaccinia virions in vitro: an electron microscopic study. J Ultrastruct Res 14: 484–496
10. Falk E (1978) Parapoxvirus infections of reindeer and musk ox associated with unusual human infections. Br J Dermatol 99: 647–654
11. Fleming SB, Blok J, Fraser KM, Mercer AA, Robinson AJ (1993) Conservation of gene structure and arrangement between vaccinia virus and orf virus. Virology 195: 175–184
12. Foerster R, Wolf G, Mayr A (1994) Highly attenuated poxviruses induce functional priming of neutrophils in vitro. Arch Virol 136: 219–226
13. Francki RIB, Fauquet CM, Knudson DL (eds) (1991) Classification and Nomenclature of Viruses. Fifth Report of the International Committee on Taxonomy of Viruses. Springer, Wien New York (Arch Virol [Suppl] 2)
14. Goebel SJ, Johnson GP, Perkus ME, Davis SW, Winslow JP, Paoletti E (1990) The complete DNA sequence of vaccinia virus. Virology 179: 247–266
15. Gordon J, Mohandas A, Wilton S, Dales S (1991) A prominent antigenic surface polypeptide involved in the biogenesis and function of the vaccinia virus envelope. Virology 181: 671–686
16. Guesdon JL, Ternynck T, Avrameas S (1979) The use of avidin-biotin interaction in immunoenzymatic techniques. J Histochem Cytochem 27: 1 131–1 139
17. Hicks BD, Worthy GA (1987) Sealpox in captive grey seals (Halichoerus grypus) and their handlers. J Wildl Dis 23: 1–6
18. Hirt P, Hiller G, Wittek R (1986) Localization and fine structure of a vaccinia virus gene encoding an envelope antigen. J Virol 58: 757–764
19. Hussain KA, Burger D (1989) In vivo and in vitro characteristics of contagious ecthyma virus isolates: host response mechanism. Vet Microbiol 19: 23–36
20. Joklik WK (1962) The purification of four strains of poxvirus. Virology 18: 9–18
21. Kearny JF, Radbruch A, Liesegang B, Rajewsky K (1979) A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J Immunol 123: 1 548–1 550

22. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
23. Lard SL, Roehrig JT, Pearson LD (1991) Differentiation of parapoxviruses by application of orf virus-specific monoclonal antibodies against cell surface proteins. Vet Immunol Immunopathol 28: 247–258
24. Lowry ON, Rosebrough NJ, Farr L, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–278
25. Lyttle DJ, Fraser KM, Fleming SB, Mercer AA, Robinson AJ (1994) Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. J Virol 68: 84–92
26. Mathiesen SD, Jorgensen T, Traavik T, Blix AS (1985) On contagious ecthyma and its treatment in muskoxen (Ovibos moschatus). Acta Vet Scand 26: 120–126
27. Mayr A, Herlyn M, Mahnel H, Danco A, Zach A, Bostedt H (1981) Bekämpfung des Ecthyma contagiosum (Pustulardermatitis) der Schafe mit einem neuen Parenteral-Zellkultur-Lebendimpfstoff. J Vet Med B 28: 535–552
28. McKeever DJ, Reid HW, Inglis NF, Herring AJ (1987) A qualitative and quantitative assessment of the humoral antibody response of the sheep to orf virus infection. Vet Microbiol 15: 229–241
29. Merril Cr, Goldman D, Sedman SA, Ebert MH (1981) Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211: 1 437–1 438
30. Mitchiner MB (1969) The envelope of vaccinia and orf viruses: an electron cytochemical investigation. J Gen Virol 5: 211–220
31. Moens U, Wold I, Mathiesen SD, Jorgensen T, Sorensen D, Traavik T (1990) Parapoxvirus papillomatosis in the muskoxen (Ovibos moschatus): genetical differences between the virus causing new outbreak in a vaccinated herd, the vaccine virus and a local orf virus. Acta Vet Scand 31: 17–25
32. Munz E, Schillinger D, Reimann M, Mahnel H (1986) Electron microscopical diagnosis of Ecthyma contagiosum in camels (Camelus dromedarius). First report of the disease in Kenya. J Vet Med B 33: 33–77
33. Murti KG, Webster RG (1986) Distribution of hemagglutinin and neuraminidase on influenza virions as revealed by immune electron microscopy. Virology 149: 36–43
34. Naase M, Nicholson BH, Fraser KM, Mercer AA, Robinson AJ (1991) An orf virus sequence showing homology to the 14K 'fusion' protein of vaccinia virus. J Gen Virol 72: 1 177–1 181
35. Robinson AJ, Balassu TC (1981) Contagious pustular dermatitis (Orf). Vet Bull 51: 771–782
36. Robinson AJ, Barns G, Fraser K, Carpenter E, Mercer AA(1987) Conservation and variation in orf virus genomes. Virology 157: 13–23
37. Rodriguez JF, Esteban M (1987) Mapping and nucleotide sequence of the vaccinia virus gene that encodes a 14-kilodalton fusion protein. J Virol 61: 3 550–3 554
38. Rosenbusch RF, Reed DE (1983) Reaction of convalescent bovine antisera with strain-specific antigens of parapoxviruses. Am J Vet Res 44: 875–878
39. Simpson VR, Stuart NC, Stack MJ, Ross HA, Head JC (1994) Parapox infection in grey seals (Halichoerus grypus) in Cornwall. Vet Rec 134: 292–296
40. Stern W, Dales S (1976) Biogenesis of vaccinia: isolation and characterization of a surface component that elicits antibody suppressing infectivity and cell-cell fusion. Virology 75: 232–241
41. Sullivan JT, Mercer AA, Fleming SB, Robinson AJ (1994) Identification and characterization of an orf virus homologue of the vaccinia virus gene encoding the major envelope antigen p37K. Virology 202: 968–973

42. Thomas V, Flores L, Holowczak JA (1980) Biochemical and electron microscopic studies of the replication of milker's nodevirus. J Virol 34: 244–255
43. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4 350–4 354
44. van Heyningen V, Brock DJH, van Heyningen S (1983) A simple method for ranking the affinities of monoclonal antibodies. J Immunol Methods 62: 147–153
45. Wilson TM, Sweeny PR (1970) Morphological studies on seal poxvirus. J Wildl Dis 6: 94–99
46. Wittek R, Herlyn M, Schlümperli D, Bachmann PA, Mayr A, Wyler R (1980) Genetic and antigenic heterogeneity of different parapoxvirus strains. Intervirology 13: 33–41
47. Yirrell DL, Vestey JP, Norval M (1994) Immune responses of patients to orf virus infection. Br J Dermatol 130: 438–443

Authors' address: Dr. C.-P. Czerny, Institut für Medizinische Mikrobiologie, Infektions und Seuchenmedizin, Veterinärstrasse 13, D-80539 München, Federal Republic of Germany.

Received May 31, 1996