Ectromelia virus persistence revisited: virus detection by *in situ* PCR after long-term infection of BALB/c mice

FELIX N. TOKA[1], IRMA SPOHR CESPEDES[1], ADA SCHOLLENBERGER[1], MAŁGORZATA KRZYŻOWSKA[1], MAŁGORZATA GIERYŃSKA[1], MIAO RU[2], MAREK NIEMIAŁTOWSKI[1]

[1]Immunology Laboratory, Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, Warsaw, Poland; [2]Hohhot Animal Quarantine Institute, Hohhot Inner Mongolia P.R. China

Abstract

We measured the extent of ectromelia virus (ECTV) persistence in experimentally infected BALB/c [H-2^d] by means of in situ PCR and report that ECTV persists in the spleen for at least 12 weeks. Importantly, ECTV maintained a low profile replication in the spleen of mice allowing it to persist considerably longer following initial infection in the absence of clinical disease. Recovery of the virus by cell culture was possible up to 8 weeks post infection. Since ECTV is an Orthopoxvirus that infects genetically susceptible mouse strains, the implication of our findings is that careful screening for this natural mouse pathogen using sensitive assays such as in situ PCR should be periodically carried out in colonies of susceptible mice. On the other hand ECTV and BALB/c [H-2^d] form a suitable study model for generalized viral infection. As such in the light of the data described here the model would suit investigations on the immune status of persistently infected animals.

Key words: *mousepox virus, ectromelia, persistence, in situ polymerase chain reaction*

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Abbreviations used: BCIP - 5-bromo-4-chloro-3-indolyl; ddH_2O - double distilled water; DLN - Draining Lymph Nodes; dpi - days post infection; ECTV - Ectromelia virus; EHV-1 - Equine herpesvirus type1; EMBO - European Molecular Biology Organization; FDA - Fluorescein diacetate; FITC - Fluorescein 5(6)-isothiocyanate; *is*PCR - in situ polymerase chain reaction; MEM - Minimum Essential Medium; NBT - nitro blue tetrazolium; PFA - Paraformaldehyde; PBS - Phosphate buffered saline; RT-PCR - Reverse transcriptase - polymerase chain reaction

Introduction

Many large DNA viruses including Poxviruses and Herpesviruses have the capability to evade the host immune surveillance (Niemiałtowski et al., 1997, Banks and Rouse, 1992, Alcami and Koszinowski, 2000) and so perpetuate their presence in the infected host. Latency is an effective process through which viruses mask their genetic material from the host's specific and non-specific defense mechanisms. There are two categories that classify the form of virus persistence in the infected host. The first form usually termed as chronic diffuse or chronic local infection, occurs when the infecting virus is present in host tissues and can be easily recovered by conventional biological assays (Boldogh et al, 1996). The second category involves integration of the viral genome into the host DNA and in most cases no viral protein production ensues, but the virus may be reactivated under favorable conditions (Six et al., 2001). Such infections are usually called latent infections (Tyler and Nathanson, 2001). However, this distinction is becoming blurred since both processes may be dependent on the cell type infected (Redpath et al., 2001).

Ectromelia virus (ECTV, mousepox virus) has been reported to suddenly cause outbreaks in mouse colonies. This sudden appearance of acute disease led some investigators to hypothesize that the virus may be latent in normal mice (Gledhill, 1962). Although several reports exist

Correspondence: Felix N. Toka, Immunology Laboratory, Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, Ciszewskiego 8, 02-786 Warsaw, Poland; tel./fax +48 22 593 60 66, e-mail: toka@alpha.sggw.waw.pl

in which ECTV has been found persistently in mice (Barski, 1960, Gledhill, 1962, , Schröeder, 1970, Walter, 1979, Shelukhina, 1979) no clear mechanisms of persistence has been put forward to explain out breaks in apparently healthy mice. The current technical advancement in molecular biology allows detection of viral DNA sequences directly in various tissues. Accordingly, it should be possible to readdress mousepox virus persistent infection and delineate the sites of persistence. If latency is involved, although quite unlikely due the characteristics of poxvirus gene expression, are any latency associated transcripts being expressed? Answers to these questions will immensely contribute to the understanding of mousepox virus immunobiology and enforce cautionary procedures in breeding susceptible mice.

ECTV causes clinical ectromelia in genetically susceptible mice and it is a classical example of generalized virus infection. In BALB/c [H-2^d] mice mousepox is usually preceded by symptoms such as swelling of the snout, ruffled coat and conjunctivitis (Fenner, 1981). Infection with ECTV in BALB/c mice can also take on a rapidly fatal form and usually mortality is high. The classical form involves a peak of characteristic skin pox lesions followed by recovery from disease. Thereafter, mice exhibit no signs of infection although, conventionally the virus can be detected in macrophages and dendritic cells 60 days post infection (dpi) (Spohr et al., 1995) suggesting that the virus can persist and therefore pose a potential hazard to mouse colonies. Our goal was to investigate the extent of ECTV persistence in BALB/c mice and to define the tissue harboring the virus after initial experimental infection followed by prolonged observation, and to apply *in situ* Polymerase Chain Reaction (*is*PCR) as a sensitive means of detecting viral DNA. The study shows that conventionally, the virus is not readily recovered from particular mouse tissue samples beginning from 45 dpi, but viral DNA sequences can be detected until 90 dpi. in the spleen by the polymerase chain reaction technique. The results described here should provide ground for further experimentation to explain the pattern of virus infection observed.

Materials and methods

Virus

Moscow strain of ECTV was used throughout the experiments (obtained from Dr. RML Buller, NIAID, NIH, Bethesda MD, USA; present address St. Louis University Health Sciences Center, St. Louis, MO, USA). Filtered spleen homogenate previously titrated in mice at 6.7 ED_{50} was stored at -80°C until use.

Mice

5-6 weeks old BALB/c [H-2^d] mice were purchased from the Children's Memorial Health Institute in Warsaw. Mice were first acclimated for 1 week then used in experiments. Thirty microliters of $1x10^{4.5}$ pfu/ml were used to infect the mice in the footpad followed by observation for 105 days. Tissue samples from infected mice were collected beginning from day 5 then day 15 and thereafter every fortnight until 105 dpi. At each stipulated interval mice were sacrificed and lymph nodes, liver, spleen, epidermis and conjunctiva removed. Respective tissue specimens from uninfected mice served as control in all experiments. Animal experiments were conducted in accordance with the requirements of the Institutional Animal Care and Use Committee at the Warsaw Agricultural University.

Virus isolation

Liver, spleen, draining lymph nodes (DLN, inguinal), epidermis and conjunctiva from infected and control mice were homogenized and filtered through 0.22 µm pores (Corning Inc., USA), then tenfold dilutions in MEM (without serum) were inoculated into Vero or BSC-1 cells grown to confluence in 96-well microtiter plates in minimum essential medium (MEM) supplemented with 10% fetal calf serum. Adsorption was carried out for 1 h at 37°C in 5% CO_2 and later the cells were washed 3 times in MEM (without serum) then incubated under the same conditions for 96 h. $TCID_{50}$ was determined by a fluorometric method as described elsewhere (Toka et al., 1997). Briefly, at 96 h post inoculation the plates were spun at 1000 rpm for 15 min. then 100 µl of medium was carefully aspirated. Fluorescein diacetate (FDA) was added at 5 µg/ml in a volume of 100 µl and plates were further incubated at 37°C in 5 % CO_2 for 45 min. Fluorescence was subsequently read at 485 nm excitation and 538 nm emission in Fluoroskan II Neonate (Labsystems, Finland). Viral titers ($TCID_{50}$) were calculated with Thompson's method as described by Levi et al. (1995).

Immunofluorescence

Tissue sections (5 µm thick) adjacent to those used for *is*PCR were first fixed in methanol at 4°C for 20 min. and washed in double distilled water (ddH_2O) then blocked against residual binding with normal mouse serum. Polyclonal rabbit anti-ECTV FITC conjugated antibody was applied to each tissue section and incubated for 1 h at room temperature. Later, tissue sections on glass slides were extensively washed in ddH2O, air dried, mounted in glycerol and examined in Olympus BX60 microscope (Olympus).

In situ PCR

For the purpose of detecting ECTV DNA in tissue samples, a fragment of p28 gene of ECTV was amplified. The rationale for selecting p28 was the lack of information on a gene unequivocally expressed during replication of ECTV *in vivo*. p28 is a highly conserved gene, it is expressed early in replication and the protein product localizes to the

cytoplasm of the infected cell. Therefore, the presence of p28 would suggest on-going virus replication in the cell. Furthermore, p28 is an important virulence factor of ECTV. Orthopoxvirus strains lacking p28 do not efficiently replicate *in vivo* and are easily eliminated by the host immune system [Brick et al., 2000, Senkevich et al., 1994, 1995]. The primers used to amplify the p28 fragment were designed with Prime, a program within the GCG software package (Oxford Molecular) made available by the European Molecular Biology Organisation (EMBO) node at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw, and synthesized by Ransom Hill Bioscience (USA) and Sigma Ark (Germany). *is*PCR was performed on frozen tissue sections. Removed organs were immediately immersed in isopentane, snap frozen in liquid nitrogen and stored at -80˚C until sectioning. 5 µm sections were cut and picked on silanated glass slides then dried under laminar airflow and finally fixed in 4% paraformaldehyde for 15 h. Next, the tissue sections were washed thoroughly in ddH$_2$O then permeabilized with 0.5 µg/ml proteinase K (Sigma, USA) for 30 min. at 37˚C (the 30 min. incubation period and proteinase K concentration were determined empirically). Later the enzyme was inactivated by immersing the tissue sections in glycine-PBS (2 mg/ml) for 5 min. and washing in ddH$_2$O. The amplification mixture contained the following: 10x PCR buffer, MgCl$_2$ 4.5 µM, dATP, dCTP, dGTP, dTTP and DIG-dUTP 200 µM (Boehringer-Mannheim, Germany), p28 antisense 5' AAT ATA TGG CAT AGA ACA CG 3' and sense 5' TCT AGT GAT ATT TTG GCA AG 3 primers at 50 pmoles each, 10 U Taq DNA polymerase (Boehringer-Mannheim, Germany), 0.001% gelatin and H$_2$O. A 50 µl volume of the amplification mix was pipetted onto each tissue section and sealed with the assembly tool (Perkin Elmer, USA) and glass slides were immediately placed in a Perkin Elmer thermal cycler (GenAmp *In situ* PCR System 1000, Perkin Elmer, USA). Initial denaturation step was performed at 94˚C for 5 min. then 94˚C 1 min, 55˚C 2 min. and 72˚C 3 min. for a total of 30 cycles. The final elongation step was set at 72˚C for 5 min.

Amplified signal detection

Digoxigenin labeled nucleotides were directly incorporated into the amplified DNA. Amplified products were detected after blocking residual binding capacity for 30 min. in blocking reagent (Boehringer-Mannheim, Germany) and staining with alkaline phosphatase conjugated anti-digoxigenin Fab fragments (7.5 U/ml) for 30 min. at room temperature in a humidified chamber. The substrate 5-bromo-4-chloro-3-indolyl (BCIP) in the presence of nitro blue tetrazolium (NBT) (Boehringer-Mannheim, Germany) was applied to the sections and slides incubated at room temperature for a maximum of 1 h (control sections) or the reaction was stopped upon appearance of color. Tissue sections were counterstained with methyl green and mounted in glycerol then examined in Olympus BX60 microscope for specific staining.

Solution phase PCR

DNA was isolated from the same samples as the ones used for *is*PCR with lysis buffer (100 mM Tris HCl pH 8.5, 0.5 M EDTA, 10% SDS, 5 M NaCl and 25 mg/ml proteinase K) for 2-3 h at 55˚C with periodical agitation. DNA was precipitated with isopropanol and dissolved in TE buffer (10 mM Tris HCl, 0.1 M EDTA, pH 7.5) at 37˚C and kept at 4˚C until PCR analysis. 20 ng of DNA were added to a 50 µl PCR mixture described in section 2.5. Taq DNA polymerase was used at 0.5 U and dNTPs were non-labeled. Controls included EHV-1 DNA, omission of ECTV-specific primer sequences, omission of dNTPs and Taq DNA polymerase. PCR integrity was checked by amplifying a house keeping gene c-myc constitutively expressed in mouse tissues was also amplified by RT-PCR from the same samples using specific primers (antisense 5' TCT CCA TCC TAT GTT GCG GTC G 3', sense 5' GGT TCA CCA TGT CTC CTC CAA GTA ACT CGG 3') (Gressens, 1994). Amplification products were visualized on 2% agarose gel with ethidium bromide.

Results

Clinical signs

Following footpad infection with 30 µl of 1x10$^{4.5}$ pfu/ml, characteristic clinical symptoms of mousepox were observed in BALB/c mice beginning from 5 dpi, mainly ruffled coat and swelling of the snout. At 15 dpi lacrimation was observed in majority of the mice. However, no amputation of limbs was observed. Beyond 15 dpi the remaining mice recovered and showed no symptoms of mousepox. Although the virus dose used was determined in preliminary studies to cause only a mild form of mousepox, about 12% of the infected mice died by day 10 pi.

ECTV antigen(s) is detectable conventionally for at least 60 dpi

Tissues collected at 5 dpi and subjected to immunofluorescence showed the presence of viral antigen in DLN, spleen and liver (Fig. 1A), unlike conjunctiva and epidermis in which no specific fluorescence was seen (data not shown). Also no fluorescence was detected in control tissue sections (data not shown). All tissue sections from infected mice at 15 dpi showed positive fluorescence including the conjunctiva and epidermis (data not shown). Immunofluorescence observed in ECTV-positive sections begun to diminish from 30 dpi and had completely disappeared by 60 dpi from the DLN and liver except for a few focal points in the spleen (Fig. 1B). No specific fluorescence was detected in all tissue sections from 75 dpi until the end of the experiment at 105 dpi.

Table 1. ECTV titer in DLN, spleen, liver, conjunctiva and epidermal homogenates at various times p.i. Titers are expressed as $\log_{10}$ $TCID_{50}$ in Vero cells. Three replicates of each tissue homogenate were assayed. ± = standard deviation

Tissue	Day post infection							
	5	15	30	45	60	75	90	105
DLN	3.0±0.12	3.50±0.33	3.00±0.13	2.79±0.30	0.87±0.51	nd*	nd	nd
spleen	2.19±0.71	3.76±0.11	3.58±0.05	3.48±0.19	1.59±0.34	0.79±0.13	nd	nd
liver	0.78±0.61	2.35±0.66	1.97±0.45	0.97±0.17	nd	nd	nd	nd
epidermis	nd	2.61±0.22	1.07±0.12	0.97±0.55	nd	nd	nd	nd
conjunctiva	nd	1.03±0.23	0.56±0.21	nd	nd	nd	nd	nd

**not detected*

Virus isolation

At 5 dpi ECTV was readily recoverable from DLN, spleen and liver but not conjunctivae and epidermis. The highest titers were found only at 15 dpi in the spleen and DLN (Table 1). Subsequent virus isolation showed a gradual reduction in virus titers from all examined tissue samples. Beginning from 45 dpi it was not possible to re-isolate ECTV from conjunctiva and 60 dpi from liver and epidermis without previous blind passages, which indicated that the virus was still present albeit at low copy number than can be conventionally detected. There was no difficulty in isolating virus from spleen up to 60 dpi. However, virus isolation from epidermis and liver collected at 45 and 60 dpi could be enhanced by applying centrifugal infection enhancement procedure. We added 2 ml of filtered tissue homogenate to a monolayer of Vero cells in 6-well plates and centrifuged at 1000 rpm for 5 min. then washed and incubated routinely. This method greatly improved recovery of virus from spleen up to 75 dpi but re-isolation trials at 90 and 105 dpi did not show replicating virus.

Validation of the in situ PCR procedure

The validation of the *is*PCR technique was performed by amplifying ECTV DNA in Vero cells previously infected with Moscow strain of ECTV at a dose of 1×10^6 pfu/ml (m.o.i. 2). After inoculation the cell culture was incubated for 48 h and later scrapped off with a rubber policeman, washed twice and counted. 50 µl of 1×10^5 cells/ml were pipetted onto silanated glass slides and further processed for *is*PCR as described in Materials and Methods. A large portion of cells was positive for ECTV-p28 DNA (Fig. 2). We also ruled out the possibility of signal leaking to uninfected cells by mixing infected and non-infected cells at proportions 1:1, 1:2 (data not shown) and 1:10. The results showed the expected ratio of infected (positive) to non-infected cells (Fig. 2 C, D).

In situ PCR detects ECTV DNA at least up to 90 dpi

*is*PCR performed on tissues isolated from mice at 5 dpi revealed very strong amplification signal particularly in DLN and spleen (Fig. 3). Detection of the signal after direct

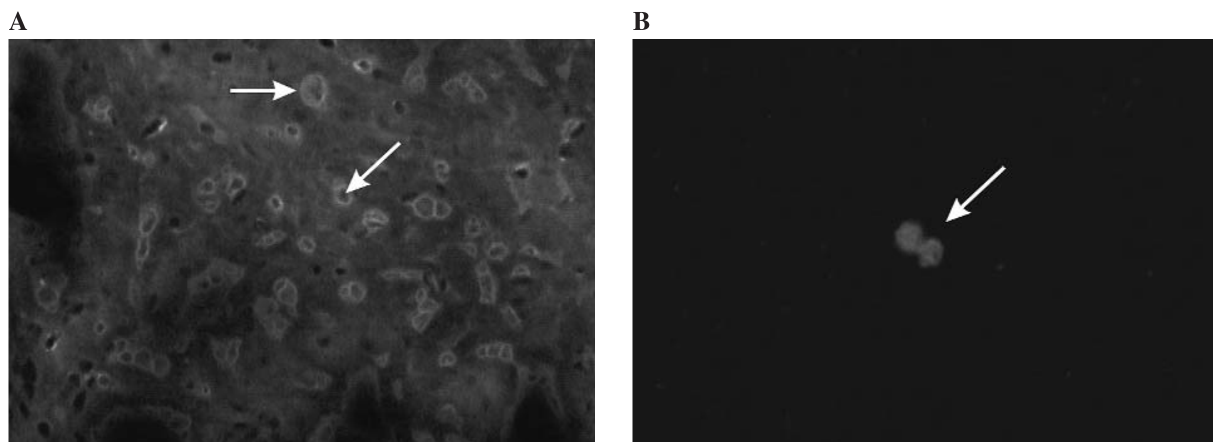

Fig. 1. Direct fluorescent antibody technique applied to tissue sections prepared from mice infected with Moscow strain of ECTV. (A) liver at 5 dpi, (B) spleen at 60 dpi. Original magnification x300. Arrows indicate positive cells

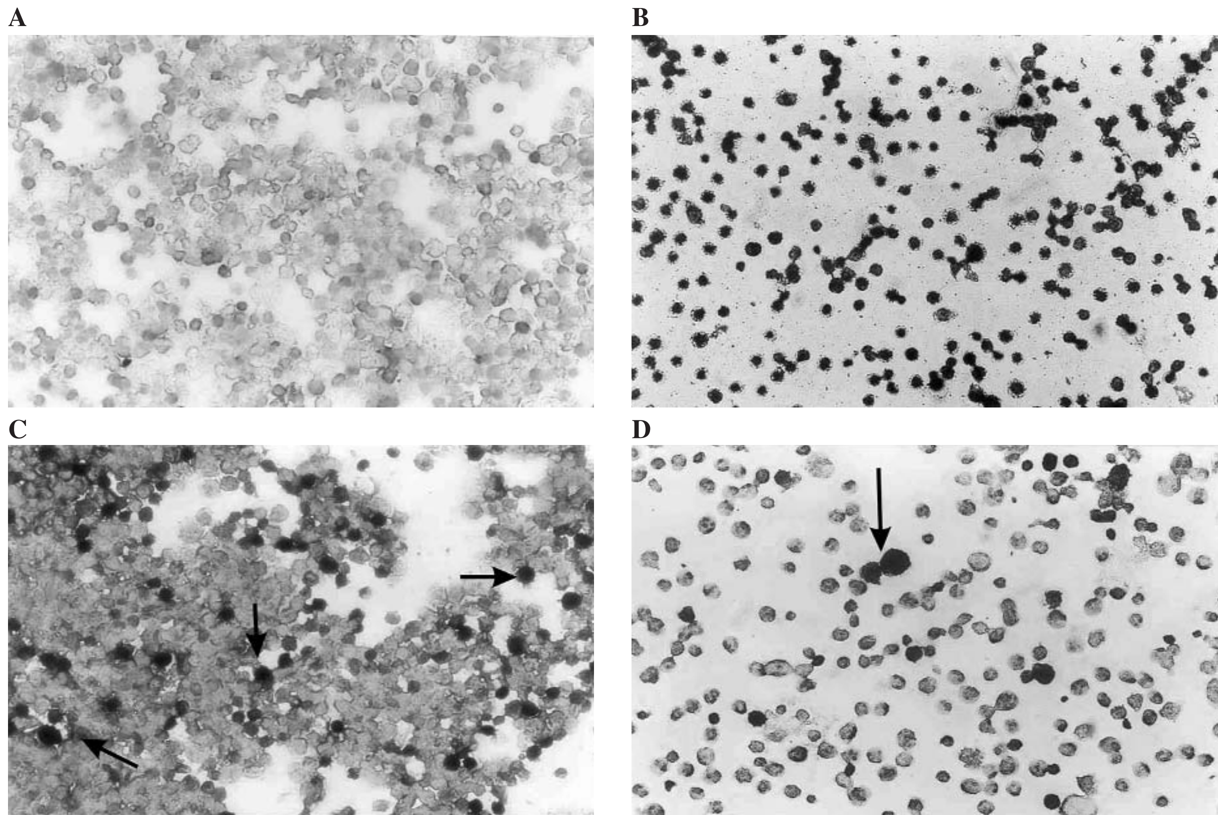

Fig. 2. *In situ* localization of ECTV DNA in Vero cells by *is*PCR. (A) non-infected negative control cells counterstained with methyl green, (B) ECTV-infected cells, (C) ECTV-infected cells mixed with non-infected cells at a ratio of 1:1, (D) ECTV-infected cells mixed with non-infected cells at a ratio of 1:10. Original magnification x300. Arrows indicate positive cells showing a dark staining - localization of anti-digoxigenin antibody labeled with alkaline phosphatase and developed with BCIP/NB

incorporation of a labeled nucleotide requires addition of enzyme-conjugated antibody and development of color with BCIP in the presence of NBT, which produces a dark purple precipitate at the site of amplification within the cell, hence the name *in situ* PCR. Labeled cells were visible as either focal or scattered cells throughout the tissue section. p28 signal was localized to the cytoplasm and in most cases overshadowed the nucleus. Abnormal tissue structure was noted, this was mainly due to destruction by the hash treatment of sections prepared from frozen tissue during the *is*PCR. *is*PCR performed without p28 specific primers did not produce any detectable signal in tissue sections prepared from ECTV-infected mice and no amplification signal was present in tissue sections from non-infected control mice. The specificity of the primers used was tested on equine herpesvirus type 1 (EHV-1) DNA as template and no amplification products were visible (data not shown). Other controls such as omission of dNTPs and Taq DNA polymerase also did not yield any product (data not shown). No amplification of p28 was observed in the conjunctiva and epidermis at 5 dpi (data not shown).

p28 amplification in tissue sections was intense compared to staining with FITC-conjugated anti-ECTV antibodies in the same tissue and time post infection. The signal was predominantly extranuclear as expected of ECTV which replicates in the cytoplasm. At 15 dpi amplification of p28 was observed in DLN, spleen and liver sections from infected mice including the conjunctiva and epidermal cells (Fig. 4). Figure 5 shows positive cells in DLN and spleen at 45, 60, and 90 dpi. *is*PCR p28-positive cells in the spleen localized most likely around periarteriolar lymphoid sheath. Such localization of other viral DNA amplification signals has been previously reported by Kim and Chae (2001).

Solution phase PCR

Interestingly, PCR in solution (Fig. 6) performed on the DNA isolated from the same tissue specimens as those used for *is*PCR did not detect ECTV p28 DNA sequence in DLN and liver beyond 75 dpi and the PCR product was absent at 90 and 105 dpi when the last samples were collected for analysis. However, ECTV DNA could still be detected in the spleen at 90 dpi. Evidently, the number of viral genome copies in the remaining organs had diminished suggesting the possibility of virus clearance from the host.

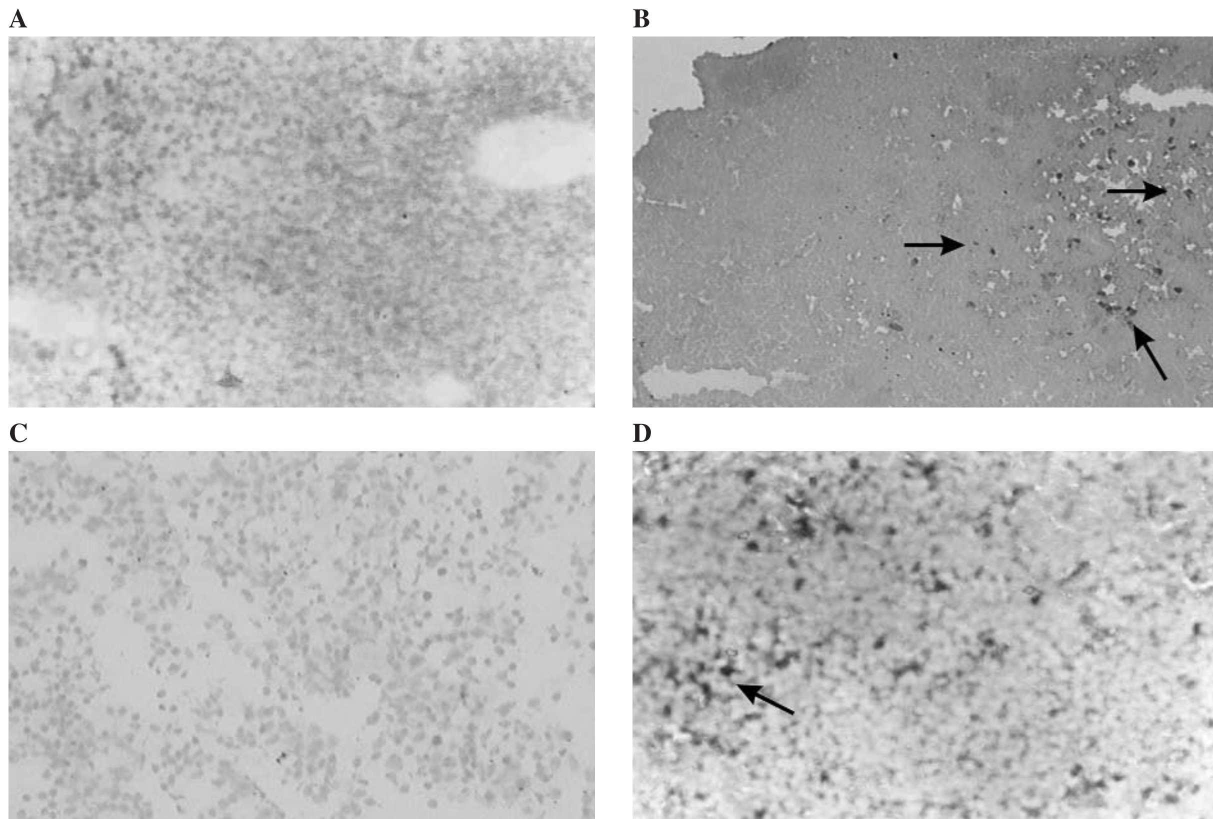

Fig. 3. *is*PCR on frozen tissue sections. (A) DLN from control mice (uninfected), (B) DLN from infected mice at 5dpi, (C) spleen from control mice at 5dpi, (D) spleen from infected mice at 5 dpi. Original magnification x300. Arrows indicate p28-positive cells

To verify the biological significance of these data, a cohort of mice infected and kept throughout the experimental period was later housed together with a new batch of uninfected mice for 5 weeks. The goal here was to investigate whether the remaining viral DNA was associated with the expression of viral proteins and viral infectivity. Out of the 5 new healthy mice housed with infected, only 2 tested positive for ECTV DNA but no clinical Ectromelia was evident.

Discussion

This study examines the extent of ECTV persistence in experimentally infected BALB/c [H-2^d] mice. We show that the virus was gradually eliminated from the host and lasted until 90 dpi. Among the examined organs the spleen was the last site at which ECTV DNA was detected. All typical forms of mousepox were seen, i.e., acute infection leading to death before 15 dpi and recovery of the majority. However, beyond 18 dpi sporadic deaths still occurred but postmortem did not attribute them to virus activity since no pathological lesions were characteristic of ECTV infection. Despite the virus load found in mice at 18 dpi the definite cause of sporadic deaths largely remains unexplained. A similar pattern of infection was reported by O'Neill et al. (1983) in other susceptible mouse strains. On the other hand the surviving mice became asymptomatic amidst ECTV persistence especially in spleen and liver. Karupiah et al. (1993) have reported the critical role played by interferons in the rapid control of ECTV infection and shortly followed by establishment of ECTV persistence at sites of infection as well as the spleen of C57BL/6 [H-2^b] mice.

The failure to readily isolate the infectious virus conventionally beginning from 60 dpi clearly indicates the reduction of virus particles required to maintain an infection. This is also evident from the difference in amplification signal intensity during the acute ECTV infection (up to 15 dpi) and the signal obtained at subsequent periods of mousepox infection. It appears that the level of viral DNA detection in *is*PCR is higher than in assays such as virus isolation in culture or solution phase PCR. The difference in detection sensitivity between *is*PCR and in solution PCR could be due to the fact that few copies of viral DNA present in the sample are diluted among other DNA not necessarily having ECTV DNA

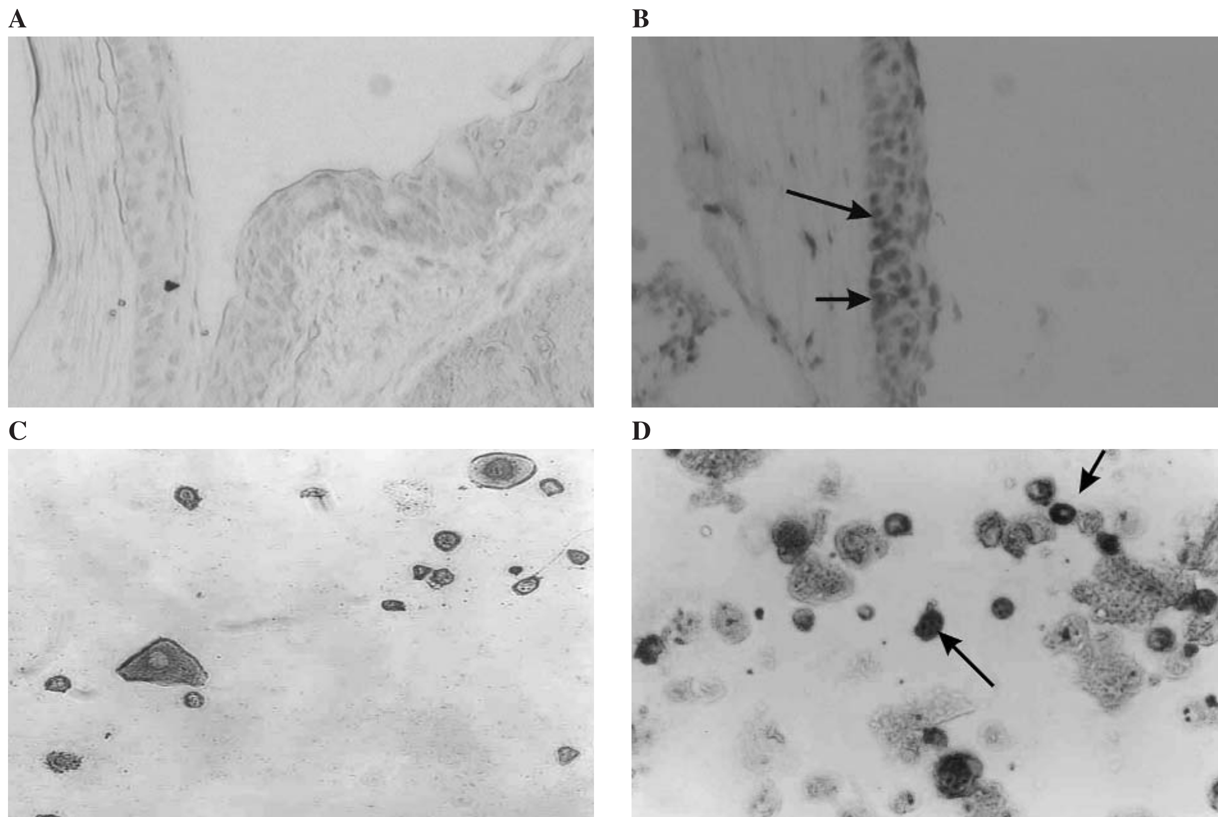

Fig. 4. *is*PCR in conjunctival and epidermal cells at 15 dpi. (A) conjunctiva of non ECTV-infected mice – negative control mice, (B) part of orbital conjunctiva from infected mice, (C) epidermal cells from control mice, (D) epidermal cells from ECTV-infected mice. Original magnification x300. Arrows indicate positive cells

sequences thus reducing the level of detection. On the other hand the cell containing viral DNA may act as a concentrator maximizing the chances of amplifying the viral DNA when the amplification reaction is performed *in situ*. It is important at this point to signalize the pitfall of *is*PCR as used in these studies. The assay is prone to occurrence of unacceptable background mainly due incorporation of the hapten-labeled nucleotide analogs into nicked DNA. As commonly accepted this is because Taq DNA polymerase has capacity to repair damaged DNA. Control reaction mixtures lacking primers should unconditionally be used to monitor the final amplification results and thus aid accurate interpretation of the data and avoid false positives (Lewis, 1996).

These studies do not address whether the partial viral genome detectable by *is*PCR at 75 and 90 dpi was associated with replication competent virus. This issue is a subject of continuing investigations.

We show that ECTV persists in the spleen of infected animals for more or equal to 90 days after infection and thereafter is cleared. This finding has a significant ecological implication since it explains the means of circulation and conservation of ECTV and likely other orthopoxviruses (Shelukhina et al., 1979) in nature. Previously we have assessed the cytotoxic activity of ECTV-specific $CD8^+$ and $CD4^+$ T cells and have shown a significant T cell burst that peaks between 12 and 15 dpi (Toka unpublished data, Toka et al., 1996). Accordingly, such a clonal burst of cytotoxic T cells that arise during ECTV infection should completely clear the virus in a relatively short time. However, this study shows that virus elimination is not that efficient, probably the virus is kept in check by T cells thus maintaining a low replication profile. We speculate that although there is a considerable clonal burst of cytotoxic T cells following infection, the precursor profile is also kept at a particular level allowing a gradual elimination of cells that harbor the virus. Allende et al. (1994) have attributed the delay in virus clearance to mutations in viral proteins causing alteration of virus epitopes. However, this can be ruled out due to very low rate of mutation in DNA viruses. Alternatively, immune evasion mechanisms usually employed by poxviruses could be at play (Alcami and Koszinowski, 2000).

The success of *is*PCR procedure on frozen tissue sections highly relies on two parameters, namely duration of tissue fixation in paraformaldehyde (PFA) and proper digestion

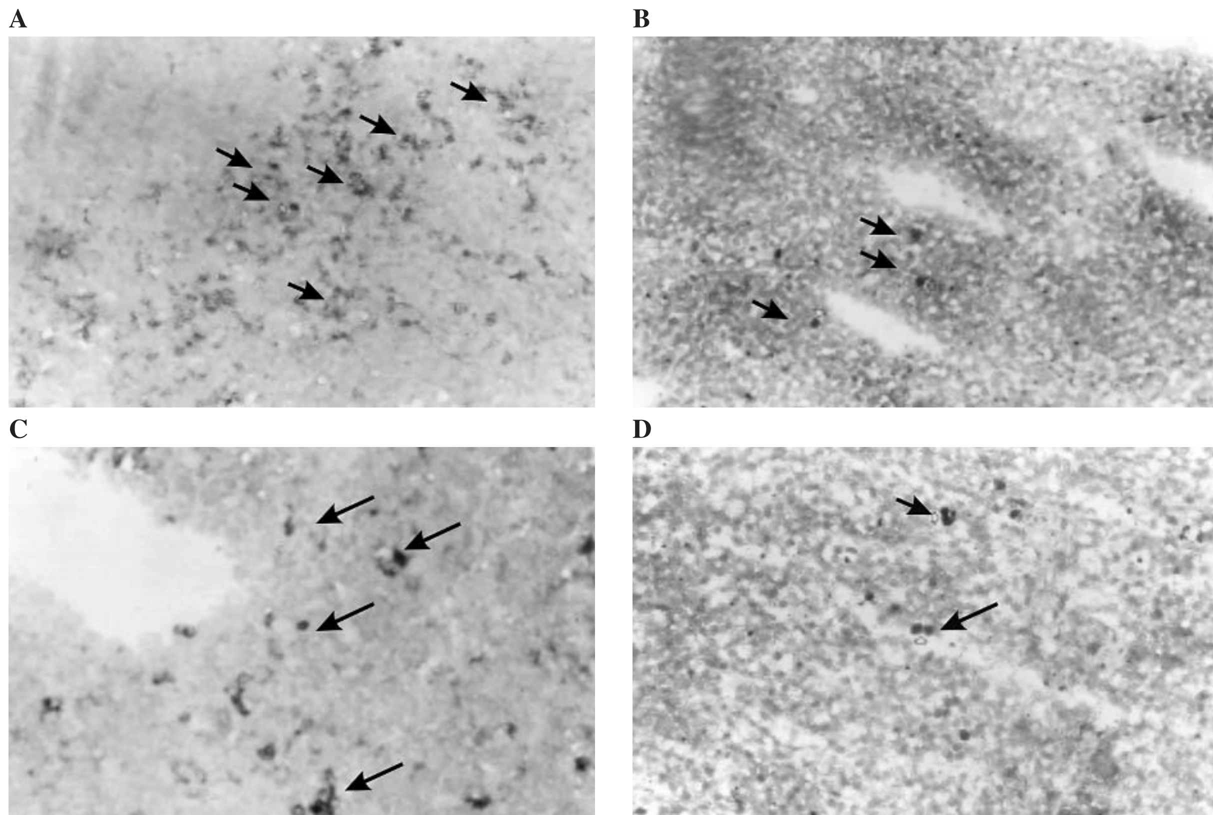

Fig. 5. *is*PCR performed on the spleen at 60 and 90 dpi and DLN at 45 and 60 dpi. (A) spleen from infected mice at 45dpi, (B) spleen at 90 dpi, (C) DLN at 45 dpi, (D) DLN at 60 dpi. Original magnification x300. Arrows indicate positive cells

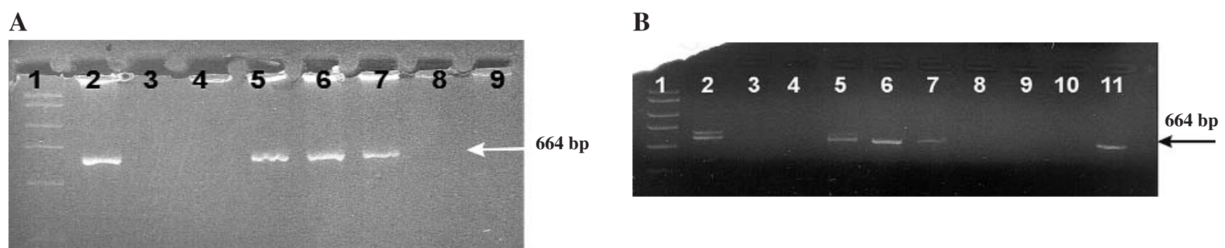

Fig. 6. Electrophoresis of PCR products from DNA isolated from DLN, spleen, liver, epidermal cells and conjunctivae of BALB/c mice infected with ECTV. (A) 5 dpi; Lane 1 – molecular weight marker 2000-300 bp, Lane 2 – ECTV DNA as positive control, Lane 3 EHV-1 DNA as negative control, Lane 4 – DNA Taq Polymerase omitted, Lane 5 – DLN DNA, Lane 6 – spleen DNA, Lane 7 – liver DNA, Lane 8 – conjunctival DNA, Lane 9 – epidermal cell DNA; (B) Lane 1 – 4 as in (A), Lane 5 – DLN DNA at 75dpi, Lane 6 – spleen DNA at 75 dpi, Lane 7 – liver DNA at 75 dpi, Lane 8 – conjunctival DNA at 75 dpi, Lane 9 – epidermal cell DNA at 75 dpi, Lane 10 – DLN DNA at 90 dpi, Lane 11 – spleen DNA at 90 dpi

with proteinase K (Nuovo, 1994). Our optimization protocols also included trypsin digestion which however, tended to overdigest the tissue sections even at lower concentration. The use of direct incorporation of labeled nucleotides results in a higher background especially in the liver. This aspect is controlled for as earlier described. However, distinction of the signal in labeled cells and unlabeled cells is clear. Positive cells had a strong signal in the cytoplasm.

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