Introduction

The shortage of human organs in allotransplantation creates a demand for alternative approaches for patients waiting for a suitable graft. A promising option is seen in the transfer of animal organs or tissues to a human recipient (xenotransplantation). Pigs are favored as organ donors, because SPF animals are available, pigs have a high reproduction rate and their organs are similar to human viscera in size and function. Besides immunological problems like organ rejection, one of the main risks is the transmission of porcine viruses and the concomitant danger a new zoonosis.

Porcine circovirus type 1 (PCV1) [1] and porcine circovirus type 2 (PCV2) [2,3] belong to the family Circoviridae. The genus Circovirus comprises PCV1 and PCV2 plus the avian viruses psittacine beak and feather disease virus (BFDV) [4,5], goose circovirus (GCV) [6], pigeon circovirus (PiCV) [7], canary circovirus (CaCV) [8] and duck circovirus (DuCV) [9]. Chicken anemia virus (CAV) [10] is the only member of the second genus, Gyrovirus. Circoviruses are characterized by small spherical...
capsids and circular single-stranded DNA genomes. They are the smallest viruses replicating autonomously in mammalian cells. With the exception of PCV1, which is not linked to a disease [11,12], all other circoviruses are pathogens and induce diseases that damage the lymphatic tissue. PCV2 is the etiological agent of post-weaning multisystemic wasting syndrome (PMWS) affecting post-weaned pigs of 4 to 8 weeks [3]. This disease was first described in Canada in 1997 [13] and is now widespread throughout important swine production areas of the world. PMWS-affected animals show fever, dyspnea, paleness and weight-loss, lymphadenopathy, hepatitis and nephritis. Microscopic findings include lymphocyte depletion and histiocytosis in the lymphoid tissue and formation of intracytoplasmic inclusion bodies [14].

Previous studies investigating the susceptibility of humans to PCV brought equivocal results. A weak reactivity of human sera with PCV was reported [15]. Seroconversion to PCV of veterinarians was not observed [16], and the infection of human leucocytes did not reveal susceptibility [17]. To deepen these studies and to investigate whether PCV infection of human cells may pose a potential risk for recipients of a xenograft, we investigated the capacity of PCV to infect human cell lines. Furthermore, human cells were transfected with viral DNA. Infected cell cultures were screened for the persistence of viral DNA, the expression and subcellular localization of viral proteins and the production of mature progeny virions to assess the risk of porcine circoviruses with respect to xenotransplantation.

Materials and methods

Amplification of PCV DNA for transfection

The plasmid pPCV2 (kindly provided by Dr D. Mahé, AFSSA, Ploufragan, France) comprises the complete genome of PCV2. It was digested with restriction nuclease SacI for 1 h at 37 °C. Viral DNA from PCV1 was isolated from the plasmid pIC1, which carries the whole genome of PCV1. A genomic fragment of PCV1 was obtained after restriction with PstI. After restriction, the 1768-bp or 1759-bp genomic fragment was extracted from a 1% agarose gel using the Perfectprep Gel Cleanup Kit (Eppendorf, Hamburg, Germany) and religated with T4-DNA ligase (New England Biolabs, Frankfurt, Germany) over night at 16 °C.

Virus production

The PCV-free PK15 cells were transfected with religated virus DNA from PCV1 and PCV2 with Effectene (Qiagen, Hilden, Germany). After two passages, confluent cells were frozen and thawed three times with shaking of the half-frozen medium in order to lyse the cells. The supernatant was centrifuged twice for 30 min at 280g and 3500g in a Heraeus Sepatech centrifuge (Heraeus, Osterode/ Harz, Germany). Ultracentrifugation followed for 1.5 h at 66 000g in a SW28 rotor. The tissue culture infectious dose (TCID)50 of the virus stock was determined using the indirect immunofluorescence assay (IFA).

Infection of human cells with PCV1 and PCV2

Previous to infection of human cells, the infectious supernatant of PCV1 and PCV2 was tested on porcine kidney cells to determine the optimal incubation period and amount of virus. As a result, infection was carried out for 5 days with PK15 cells growing in 24-well plates using 20 μl of the PCV1 stock with a TCID/50 of 10^7 and 10 μl stock of PCV2 with a TCID/50 of 10^8 and a multiplicity of infection (m.o.i.) of 3 × 10^-2 per cell. Higher virus concentration and longer incubation time did not improve infection rate of PK15 cells (data not shown). Infection of human cells was performed using similar conditions.

Two parallel sets of infections were performed. One day before infection, cells were seeded on plastic 8-whole-chamber slides (Nunc, Naperville, IL, USA). PK15 cells were used as a positive control. Five days after infection, the first batch of transfected cells was investigated by IFA to detect expression of PCV-specific antigen indicative for a PCV-infection. The second batch of PCV-infected cells was cultivated until 6 weeks after infection. The supernatant was sterility filtered and subsequently applied to PCV-free PK15 and human cells. Additionally content of virus DNA was determined by TaqMan polymerase chain reaction (PCR).

Maintenance of cells

Porcine PK15 (ATCC CCL-33) and human adherent cell lines (ATCC CCl-13), FL (ATCC Chang Liver CCL-62), RD (ATCC CCL-136), Ma23 and RH (lung fibroblast and human kidney cells, kindly provided by Prof. Pauli, Robert Koch Institut, Berlin, Germany), Caco (ATCC HTB-37), Hep2 (ATCC HB-8065), 293 (ATCC CRL-1573), HeLa (ATCC CCL-2) and human suspension cell lines Jurkat (ATCC TIB-152), C8166 (ECACC 8805 1601), CCRF-CEM (ATCC CCL-119), H9 (ATCC HTB-176), Wil2.NS.6TG (ECACC 93031001), THP1 (ATCC TIB-202), U937 (ATCC CRL-1593) and Molt4 (ATCC CCR-420).
CRL-1582) were grown in the appropriate culture medium, as indicated by ATCC. Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy blood donors after 1 : 2 dilution with phosphate-buffered saline (PBS) by Ficoll-Paque Plus (Amersham Pharmacia, Upsala, Sweden) density gradient centrifugation (400 g, 40 min, 20 °C). Cells harvested from the interphase were washed twice in PBS at 300 and 250 g for 10 min at 20 °C and the pelleted cells were resuspended in RPMI-1640 with 10% FCS and 2 mM glutamine (ICN, Costa Mesa, Roswell Park Memorial Institute, CA, USA) and 100 μg/ml streptomycin and 100 U/ml penicillin (Biochrom, Berlin, Germany). The cell counts were performed using a Casey Counter (Schaerfe System, Reutlingen, Germany). 

Transient transfection

Adherent cells were transfected with Effectene (Qiagen, Hilden, Germany). 60% confluent grown cells were transfected with 200 ng relegated DNA in 24-well plates or with 300 ng DNA in 12-well plates. To monitor the transfection efficiency, cells were transfected with plasmid pEGFP expressing the green enhanced fluorescence protein (GFP, BD Bioscience, Palo Alto); the ratio of transfected to non-transfected cells was be determined by analysis in a fluorescence microscope. Porcine PK15 cells were transfected with DNA of PCV1 or PCV2 as a positive control, mock-transfected PK15 cells were used as a negative control.

Harvesting transfected cells and DNA extraction

After transfection, adherent cells were cultivated for 1.5 month. After every passage, cells were washed with PBS and DNA was isolated from an aliquot using the Qiagen DNeasy tissue kit (Qiagen). Concentration of the extracted DNA was determined fluorometrically (TD360, Turner Designs, Sunnyvale, CA, USA).

Conventional PCR to monitor virus persistence

To investigate persistence of PCV1 and PCV2 in human and porcine control cells, a PCR was performed. Primer pair F197 5'-tgttcaaccttaa taacctctcg and B198 5'-gtctgaccttccgtcatctc were applied for the detection of PCV1, while F199 5'-cacatcgagaagaaagaa and B200 5'-ctctgacggaactcactee were used for the detection of PCV2. DNA was amplified with 12 min, 95 °C; 40x [20 s, 95 °C; 20 s, 55 °C (for PCV1 detection) or 58 °C (for PCV2 detection); 45 s, 72 °C] 10 min, 72 °C. PCR products (431 bp in size for PCV1, while a 168 bp fragment was seen for PCV2) were separated by electrophoresis on a 1% agarose gel.

TagMan PCR

Real-time PCR was performed as described recently [18].

Indirect immunofluorescence assay

Cells were grown on glass microscope slides and on 8-chamber microscope slides with a diameter of 15 mm in 24-well plates. Three days post-transfection, the cells were first washed with PBS and then fixed with 4% paraformaldehyde (Roche, Mannheim, Germany) for 15 min at room temperature. After washing with PBS cells were permeabilized with 0.1% Triton 100 (Roche) for 10 min at room temperature. After washing three times, cells were blocked with 3% fetal calf serum and 0.1% non-essential amino acids (Gibco) and 1% l-glutamine (ICN).

Replication assay

The capacity of PCV1 and PCV2 to replicate in human cells was tested with a newly developed reporter gene assay in 293 and Hep2 cells [19]. For this purpose, cells were cotransfected with three plasmids. Plasmid pRL16 (or pRL16.2) carried the origin of replication of PCV1 (or PCV2), cloned
into plasmid pGL3-promoter (Promega, Madison, WI, USA). pGL3-promoter expresses the luc gene constitutively under control of the late SV40 promoter. The second plasmid, pORF4 and pSVL-rep(PCV2) express the replicases of PCV1 and PCV2 and the third plasmid, pRSV-ßGal was used to standardize for transfection efficiency. Replication of luc-plasmids occurs, when the cognate replicase is expressed. Replication leads to an increase in copy number and a concomitant higher level of Luciferase (Luc) expression. Luc expression is measured and compared with basal expression in the plasmid without the origin of replication. To normalize for variation of the transfection efficiencies, standardization of this assay is achieved by dividing the Luc units through the Gal units. Each assay was performed three times in duplicates, mean values were calculated.

In case of examination of the replication activity of PCV in human cell lines, 100 ng of plasmid pRL (ori PCV1) plus 100 ng pORF4 (Rep PCV1) or pSVL-rep (PCV2) were cotransfected with 50 ng of pRSV-ßGal into 293 and Hep2 cells. After 48 h, cells were washed and lysed, extracts were measured for Luc and Gal activity as described previously [19].

Results

PCV-infection of human adherent and suspension cell lines

To investigate whether human cell lines are susceptible for PCV, cells were infected with PCV1 and PCV2. Nine different adherent human cell lines (Chang liver, FL, 293, Hep2, RH, CaCo, Hela, Ma23 and Rd) were either transfected with religated DNA of PCV1 and PCV2 or infected with PCV. As suspension cell lines are not suitable for transfection, the cell lines Wil2, THP1, Jurkat, Molt4, C8166, CEM, U937 and H9 were only infected with PCV1 and PCV2. Additionally, human PBMCs and four animal cell lines (Vero/monkey, PG-4/cat, CTL-6/mouse and RAT-2/rat) were included in the study.

Three days after treatment with virus or viral DNA, cells were tested for expression of PCV-specific proteins using the IFA. Localization of viral proteins was determined by confocal laserscan-microscopy. Initially PCV-positive cells were maintained for 6 weeks, presence of PCV DNA in the infected cells was continuously monitored by PCR. TaqMan PCR was used to determine the amount of PCV DNA in the supernatant. Excretion of infectious PCV particles was analyzed with IFA after transferring the supernatant to PCV-free porcine and human cell lines. Porcine PK15 cells are permissive for PCV and were used as a positive control in all studies.

Detection of PCV in cell lines

Five days after transfection of adherent cell lines with DNA of PCV1 and PCV2, a PCV-specific PCR was performed. PCV1 DNA was detected in 293, RH, Chang liver and Hep2 cells as well as in the positive control PK15 (Fig. 1A). This result was seen repeatedly until the end of the experiment after a 6-weeks period (Fig. 1B). Only a weak PCR signal was seen in Hela cells, while CaCo, FL, Ma23 (data not shown) and mock-transfected control cells were negative. IFA revealed presence of viral antigen in 293, Hela, RH, Chang liver and Hep2 cells (Fig. 2A, panel 1 to 5). Cell morphology was examined with a light-microscope and did not reveal any particularities.

The PCV2 DNA was amplified from PCV2-transfected 293, Hela, Hep2, RH and Chang liver cells (Fig. 1C). Likewise, expression of viral antigen was seen in all cells (Fig. 2A, panel 6 to 10). No positive PCR signal was seen with CaCo, FL, Ma23 and in the mock-transfected control cells (data not shown). After 2 weeks, the PCV2 signal was lost (Fig. 1D). A CPE was seen in PCV2-transfected cells 3 d.p.i., the form of the cells was altered from stretched to round and dead cells and cell debris was seen in the supernatant. This effect was very pronounced in 293 and Hep2 cells. PK15 cells transfected with PCV2 DNA did not show a cytopathogenic effect. To investigate whether human cell lines can internalize PCV particles and initiate an infectious cycle, the cell lines were infected with PCV1 and PCV2 at a m.o.i. of 3 × 10⁻² per cell. Positive signals in IFA after infection with PCV1 were seen in Hep2, 293 and Chang liver cells (Fig. 2B, panel 1 to 4), while no signals were observed in FL, RH, CaCo, Hela and Ma23 cells (data not shown). After PCV2-infection, only Rd cells showed fluorescence in IFA (Fig. 2B, panel 5 and 6). Compared with infection efficiency in PK15 cells, number of PCV-positive human cells was low, although the same m.o.i. has been used for all cell lines.

PCV in vivo is often found in cells derived from the macrophage/monocyte lineage. Therefore, human suspension cell lines Wil2, THP1, Jurkat, Molt4, C8166, CEM, U937, H9 and human PBMCs were infected with PCV1 and PCV2. The porcine lymphoblastoid cell line L23 was used as a positive control, mock-infected cells as a negative control. Expression of PCV-encoded protein was neither observed in any of the human cell lines nor in PBMCs, while fluorescence was seen in porcine L23 cells (data not shown).
In addition, four animal cell lines were infected with PCV and investigated for expression of viral proteins in IFA. The IFA showed a fluorescence signal in simian Vero cells (Fig. 3B, panel 4), while murine cell lines RAT-2 and CTL were negative, as well as feline cell line PG4 and mock-infected control cells (data not shown).

Localization of viral protein

IFA was performed with antisera raised against PCV1 or PCV2 virions, which react with the Cap protein (T. Finsterbusch, pers. comm.). In PCV-permissive PK15 cells several distinct patterns of fluorescence were observed. In some cells, fluorescence occurred in the complete nucleus, in others in the nucleus with nucleoli spared or fluorescence was restricted to the cytoplasm (Fig. 3A, panel 1 and 4). In contrast to the observation in permissive cells, investigation of subcellular localization of the Cap protein in PCV-infected human cells revealed, that protein was accumulated punctiformly in the nucleus (panel 2, 3, 5 and 6). Fluorescence in the cytoplasm was not observed in the human cells or in Vero cells (Fig. 3B, panel 4).

PCV1 and PCV2 replicates in human cells

A replication assay was used to test the capacity of PCV to initiate its DNA replication in human and animal cell lines. For this purpose, Hep2, 293, Vero, PG4 and Rat-2 cells were cotransfected with a plasmid carrying the origin of replication of PCV1 (pRL16.1) or PCV2 (pRL16.2) and a constitutively expressed luc gene and a second plasmid expressing the rep gene products of

Fig. 1. Detection of PCV-DNA in transfected human cell lines: detection of PCV1 DNA in cells transfected with religated viral DNA of PCV1. The results of the amplification 1 week (A) and 6 weeks (B) after transfection of the cells are shown. A: M-marker, (1) mock-transfected PK15 cells, (2) PCV1-transfected PK15 cells, (3 to 7) mock-transfected 293, Hela, Hep2, RH and Chang liver cells, (8 to 12) PCV1-transfected 293, Hela, Hep2, RH and Chang liver cells. B: M-marker, (1) mock-transfected PK15 cells, (2) PCV1-transfected PK15 cells, (3 to 6) mock-transfected 293, Hep2, RH and Chang liver cells, (7 to 10) PCV1-transfected 293, Hep2, RH and Chang liver cells. C and D: detection of PCV2 DNA in cells transfected with religated viral DNA of PCV2. The results of the amplification 1 week (C) and 6 weeks (D) after transfection of the cells are shown. C: M-marker, (1) mock-transfected PK15 cells, (2) PCV2-transfected PK15 cells, (3 to 7) mock-transfected 293, Hela, Hep2, RH and Chang liver cells, (8 to 12) PCV2-transfected 293, Hela, Hep2, RH and Chang liver cells. D: M-marker, (1) mock-transfected PK15 cells, (2) PCV2-transfected PK15 cells, (3 to 6) PCV2-transfected 293, Hep2, RH and Chang liver cells.
PCV1 (pRep-PCV1) or PCV2 (pRep-PCV2). Replication of the luc-plasmid with the origin of PCV occurs, if the replicase is produced in trans. Consequently, this will lead to an increase in the copy number of the luc-plasmid, which can be monitored [19].

Fig. 2. Expression of PCV-encoded proteins in human cells. A: results of the indirect immunofluorescence assay 3 days after transfection of PK15 (1), Hep2 (2), Chang liver (3), 293 (4) and RH (5) cells with religated viral DNA of PCV1 and after transfection of PK15 (6), Hep2 (7), Chang liver (8), 293 (9) and RH (10) cells with religated viral DNA of PCV2. Cells were investigated with a confocal laserscan microscope (Zeiss LSM 510) at a 10-fold magnification. B: The results of the IFA 5 days after infection of PK15 (1), Hep2 (2), 293 (3) and Chang liver (4) cells with PCV1 and after infection of PK15 (5) and Rd (6) cells with PCV2 is shown. Cells were investigated with a cLSM at a 10-fold magnification.
When 293 cells were transfected with pGL3b, carrying a promoter-deprived luc gene, no endogenous Luc activity was detected (Fig. 4, column 1). When pRL16.1, carrying an active luc gene plus the origin of PCV1, was cotransfected with the vector pSVL, a basal Luc/Gal activity of 0.1 was detected (column 2). The same plasmid combined with Rep(PCV1)-expressing plasmid pORF4 resulted in an increase of Luc/Gal activity to 0.6 (column 3). The same effect was observed for pRL16.2 and pRep-PCV2 (columns 4 and 5). A similar result was obtained, when Hep2 cells were used for transfection, while simian and murine cell lines Vero, PG4 and Rat-2 did not support replication of PCV (data not shown).

The PCV infection and transfection of human cell lines and Vero cells is non-productive, although DNA can be detected.

To address the question whether virus particles have been excreted from PCV-infected and transfected human cells, supernatant of these cells was sterile filtered and examined by TaqMan PCR for presence of PCV DNA after 3 weeks of cultivation. TaqMan PCR revealed copy numbers of $1 \times 10^5$ PCV1 molecules and $6 \times 10^7$ PCV2 copies per µl supernatant (Fig. 5A and B). To examine whether infection of human cells led to production of functional viral particles and whether the infection can be transferred to uninfected cells, virus-free human cells were infected with 150 µl supernatant and incubated for 5 days. Examination of the cells by IFA did...
neither reveal a signal in human cell lines nor in porcine PK15 cells infected with the human cell derived supernatant (data not shown). Fluorescence was only observed in the positive control, in which a PK15 derived supernatant was passed onto virus free PK15 cells. This result indicates that the supernatant of PCV-infected human cells contains no infectious virus particles. This result was supported by an electron microscopic (EM) analysis of the supernatant, which did not reveal existence of viral particles (data not shown).

Discussion

In order to assess the possible transmission of PCV to a human acceptor of porcine tissues or organs,
this study investigated whether human cells are susceptible to PCV in vitro. It was tested whether PCV initiates an infectious cycle in human cells by transfecting PCV-DNA into suitable human cells and by infecting a broader panel of human and mammalian cells with PCV. Effect of this treatment was controlled at different levels: (i) presence and stability of the introduced DNA was tested by PCV-specific PCR, (ii) expression and localization of viral proteins was investigated by IFA and confocal microscopy, (iii) a replication assay was used to test replication of the virus genome, (iv) presence of PCV DNA and virus particles in the supernatant derived from the human cells was investigated by TaqMan PCR and EM diagnostic and (v) the biological activity of the supernatant was tested by inoculation of PCV-free cells and subsequent IFA.

After transfection, Chang liver, 293, Hep2, Hela and RH cells maintained PCV1 and PCV2 DNA and expressed the viral antigen as well. PCV2 DNA was lost after 2 weeks, while PCV1 persisted in all cells until the end of the 6-week experimental period. Expression rates of viral proteins were comparable with those seen in PK15 cells after transfection of the PCV genome. A cytopathogenic effect (CPE) was observed in PCV2-transfected human cells, but not in PCV1-infected cells. This effect was investigated in detail in a concomitant study and is due to the induction of apoptosis (K. Hattermann, C. Roedner, A. Mankertz, unpubl. data). In contrast to the results obtained by transfection of PCV-DNA, infection with PCV1 was not observed in RH and Hela cells. Besides Rd cells, no human cell line was susceptible to PCV2. Compared with infection of the porcine control cells PK15, infection efficiency was low in all cell lines investigated, although the same virus titer was applied in all experiments.

As PCV is seen in vivo mainly in cells deriving from the monocytes/macrophage line, eight human cell lines of this type were tested. In none of these suspension cells was PCV DNA or antigen visible, indicating that they are non-permissive for PCV. Infection of human PBMCs was not possible either, corroborating earlier results [15].

This result implies that PCV1 can be better propagated in vitro in human cell lines than PCV2, because it infects more cell lines than PCV2 and moreover, PCV2 induces a CPE. In addition, the data suggest that RH and Hela cells lack a factor or the receptor essential for infection with PCV. This factor may be the receptor or an integral membrane protein that is essential for antireceptor-binding or viral import. This molecule might be expressed in a reduced way or changed in its molecular architecture, so that the human cellular receptor and the viral capsid do not match completely. To investigate this hypothesis, it is necessary to identify the receptor for PCV.

By IFA and confocal microscopy, the Cap protein was seen either in the nucleoli, in the
complete nucleus or also in the cytoplasm of PCV-permissive PK15 cells (T. Finsterbusch, pers. comm.), while an punctiform accumulation of the Cap protein in the nucleus of the human cells, suggests exclusive localization in the nucleoli. Targeting of viral proteins to the nucleoli is not an uncommon feature [20,21]. These observations resembled those seen in PK15 cells transfected with plasmids expressing Cap fused to fluorescent proteins, i.e. experiments in which the context of a viral infection was missing (T. Finsterbusch, pers. comm.), because in these experiments, the signal was also restricted to the nucleolus. This finding suggests that protein transport or virus assembly of PCV may be perturbed in non-permissive human cells.

Replication of PCV was tested with an assay, in which replication is seen as the increase of the copy number of a reporter gene. PCV1 and PCV2 replicated in human 293 and Hep2 cells, while replication was not observed in cells of other species (rat, cat, monkey). TaqMan PCR detected $10^5$ to $10^7$ copies of the PCV genome per µl supernatant of the human cells. This method does not discriminate between naked DNA and DNA from virions, investigation by electron microscopy did not reveal viral particles. Inoculation with supernatant from PCV-infected human cells did not transmit the infection to PCV-free human cells. All these experiments indicate that virus particles are not produced and that the DNA in the supernatant may be derived from dead cells. The higher number of virus DNA found in the supernatant of PCV2-infected human cells may be attributed to the observation that PCV2 induces a CPE in infected human cells, while PCV1 does not.

In summary, this study shows that PCV-infection of human cells is non-productive. When compared with PK15 cells, PCV can enter human cells at reduced rates, which render these cell lines semi-permissive for PCV-uptake. A search for PCV-specific antibodies in human sera revealed a positive reaction when sera of patients affected by a fever of unknown cause were investigated [15], but the kinetics of this reaction made it likely that the reaction was due to a cross-reacting epitope. Investigation of sera of humans exposed to pigs was negative [16]. Taken these studies into account, transmission of PCV to humans under natural conditions seems not to a regularly occurring event. Nevertheless, the result of this in vitro study should not be seen as an all-clear to the potential threat of a new circoviral zoonosis in xenotransplanted patients. Cultivation of PCV in human cells for a longer time may give the virus and the host the opportunity for adaptation, a process that may finally end in the formation of infectious particles. Therefore, further studies should be performed including the infection of primary human cells, monkeys or primates.

Acknowledgments

We thank Ms Petra Kurzendoerfer for technical assistance. K.H. was funded by a PhD fellowship of the Robert Koch-Institut, C.S. was supported by the Sonnenfeldstiftung Berlin and the NaFoeg programme of the free University Berlin, C.R. received a grant of the ‘Dr Georg und Ingeborg Scheel Stiftung’. This work was supported by the European Union (Project number QLK2-CT-1999-00307) and the Deutsche Forschungsgemeinschaft (MA 2126/2-1).

References


