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Rat cytomegalovirus replication in the salivary glands is exclusively confined to striated duct cells

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Abstract The salivary gland is the preferred organ for cytomegalovirus (CMV) replication and viral persistence. In order to identify the nature of infected cells and to study viral replication in more detail, several experiments were conducted. Using the rat CMV (RCMV) model, acutely infected young adult rats (6 weeks of age) and new-born rats (3 days of age) were infected, and submandibular, parotid and sublingual glands were harvested at different time points after infection. For identification of the nature of infected cells, immunohistochemistry, in situ hybridisation and electron microscopic techniques were used. In young adult animals, the submandibular gland was the preferred organ for RCMV replication. The parotid and sublingual glands contained fewer viruses than the submandibular gland. In contrast, in new-born rats, the main site of RCMV replication was the sublingual gland, while the submandibular and parotid glands contained low amounts of virus. No virus could be detected in the parotid glands. In all glands of RCMV-infected animals, the infection was exclusively confined to striated duct cells. Infection resulted in a cellular inflammatory response which was mostly located in the interlobular duct region, whereas only few inflammatory cells were found in the neighbourhood of infected striated duct cells. This phenomenon may contribute to the long persistence of the virus in this organ.

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Introduction

Cytomegalovirus (CMV) is one of the most common viruses affecting humans. Infection of the immunocompetent host is usually associated with mild disease or asymptomatic infection. After primary infection, the virus persists in its host in a latent state from which it can be reactivated, as was shown for human CMV (HCMV) [7, 8, 15], murine CMV (MCMV) [41] and rat CMV (RCMV) [14].

The rat model provides an appropriate model for HCMV, since RCMV has a virion structure similar to HCMV and the pathology it induces both in vitro and in vivo is similar to the HCMV-induced pathology [16, 40, 46]. Infection of immunosuppressed rats with RCMV results in a generalised infection with the presence of infectious virus in most organs, such as liver, spleen, heart, kidney and lungs [46]. In these organs, infectious virus is already detectable at day 3 post-infection (p.i.). Within these organs, the virus is found in many cell types, including brown fat cells, endothelial cells and monocytes/macrophages. After this period – the so-called acute phase – which lasts for 1–2 weeks, no infectious virus is detectable in these organs. An exception are the salivary glands, where the virus persists for a long period, i.e. several months up to 1 year p.i. [12, 13]. The persistence of the virus in this organ is characteristic for RCMV and is believed to be the principal route by which the virus is spread within the population of susceptible hosts. In summary, in the salivary glands, RCMV infection is characterised by two main properties: (1) infectious virus can be detected at a later time point than in all other organs, starting at day 7 p.i. and (2) infectious virus is produced in very high concentrations over a long period of time.

The tropism of this virus for the salivary glands and the long-term shedding from this organ is one of the

main characteristics of all CMVs. During evolution, the virus has been adapted to this target organ, as shown by studies using deletion mutants of the virus [2, 18, 32, 43]. In rats and mice, infection with deletion mutants can result in a generalised infection with the presence of the virus in internal organs but without the infectious virus and virus-infected cells in the salivary glands. This indicates that the virus harbours within its genome the genetic code for proteins/antigens that are necessary for infection of and replication in salivary glands.

Over the years, many research groups have studied the course of CMV infection in both humans and animal models. Although the salivary gland is one of the main organs involved in replication of CMV and spread of the virus within the population, little is known of the cells that are infected and are responsible for the production of the infectious virus.

To study RCMV infection of the salivary glands more thoroughly and to identify the nature of infected cells, we injected RCMV into the peritoneal cavity of young adult rats and followed the infection in the salivary glands. It is known that during the first weeks after birth, the rat salivary glands are still developing. Therefore, we tested whether the age of the host is of importance for the infection of salivary gland cells. To study this, we included acutely infected new-born animals in this study and followed the infection in their salivary glands.

Materials and methods

Animals, virus and immunosuppression

Inbred male-specific pathogen-free (SPF; according to [42]) Lewis rats were used in all experiments. The animals were purchased from the Department of Experimental Animal Service at the University of Maastricht, The Netherlands. The animals were infected at the age of 3 days (new-born rats) or 6 weeks (young adult rats). The RCMV inoculum was derived from submandibular/sublingual salivary gland homogenate obtained as described previously [12] and was intraperitoneally (i.p.) administered at a dose of 1×10^4 and 1×10^5 plaque forming units (PFU), respectively [46]. A negative salivary gland homogenate was used for mock infection. CMV infection is known to be enhanced by immunosuppression. Therefore, the 6-week-old rats were immunosuppressed using 5 Gy total body irradiation (TBI) [46]. New-born rats have an immature immune system and were considered "naturally" immunosuppressed.

Prior to the experiments, permission was obtained from the Ethical Commission for Laboratory Animals of the University of Maastricht, Maastricht, The Netherlands, and all experiments were performed according to the principles of laboratory animal care (NIH publication no. 85–23, revised 1985) and the Dutch Law on Laboratory Animals.

Experimental design

The 6-week-old animals were divided into three groups. The first group (n=5) received immunosuppression and was subsequently infected with RCMV. A second group consisted of immunosuppressed mock-infected animals (n=3), while a third group consisted of immunocompetent (non-irradiated) RCMV-infected rats (n=3). The rats were sacrificed at 7, 14 and 28 days p.i. New-born rats (n=3) were infected at an age of 3 days and were subsequently sacrificed at 21 days p.i. At the time of sacrifice, the submandibular, parotid and sublingual glands of young adult and new-born rats were sampled and processed for histological analysis and assessment of infectious virus. None of the infected rats showed symptoms of CMV disease as was defined previously [46].

Immunohistochemistry

Tissue samples were fixed in a phosphate-buffered 3.7% formaldehyde solution for 24 h and subsequently embedded in paraffin. RCMV-induced antigens were detected in 4-µm tissue sections using a mouse monoclonal antibody (mAb) 8 directed against an early nuclear RCMV antigen [17]. For evaluation of the inflammatory response, mAbs ED-1, W3/13 and 3.2.3 were used, with specificity for monocytes/macrophages [20], T lymphocytes [10] and natural killer (NK) cells [19], respectively. Positive cells were visualised using diaminobenzidine (DAB) substrate, and slides were counterstained with haematoxilin and eosin.

For inflammatory assessment, positive cells in five representative high power fields (HPF, 400× magnification) were counted. The presence of inflammatory cells was assessed semi-quantitatively. Absence of cells was scored as –; sporadic cells as ±; small numbers of cells as +; considerable number of cells as ++; high numbers of cells +++. Images of tissue sections were analysed using a computerised morphometric analysis system (Qwin Software, Leica, Cambridge). Cellular and nuclear area were expressed as mean±SEM.

In situ hybridisation

Detection of RCMV DNA was performed using the previously described in situ hybridisation (ISH) technique [46]. In short, paraffin-embedded sections (4 µm) were dewaxed and proteolytic digestion was carried out (5 min, 37°C) with pepsin (2 mg/ml). Denaturation (80°C for 5 min) was followed by hybridisation with the DNA probe overnight at 42°C. The DNA probe consisted of the *Xba*I fragments C, D and E labelled with biotin 11-dUTP [33]. Positive cells were visualised with DAB substrate. The ISH-stained sections were used for viral load assessment since ISH is more sensitive than immunohistochemistry, as described previously [25].

Ultrastructural analysis using electron microscopy

Rats were anaesthetised by means of injection with 0.1 ml pentobarbital (6%)/100 g body weight i.p. Subsequently, rats were perfused with 0.1 M Na-cacodylate buffer + 2% polyvinylpyrrolidone (PVP; MW 40,000), pH 7.4, for 3 min (flow rate 15 ml/min), followed by perfusion fixation using fixative containing 4% paraformaldehyde, 2% glutaraldehyde and 0.2% picrin-acid in 0.1 M Na-cacodylate buffer, pH 7.4. After perfusion, salivary glands (submandibular, sublingual and parotid) were removed and fixed overnight using the same fixative. Thereafter, tissues were washed with 0.1 M Na-cacodylate buffer, pH 7.4, containing 6.8% sucrose (three 5-min washes) followed with another 2-h fixation in 1% OsO_4 and 1.5% $K_4Fe(CN)_6$ in 0.1 M Na-cacodylate buffer at 4°C. Again, tissues were washed in 0.1 M Na-cacodylate buffer, pH 7.4, containing 6.8% sucrose (three 5-min washes) at room temperature and subsequently dehydrated in 30%, 50% and 70% EtOH (10 min each) followed by 2 h in 100% EtOH. Tissues were embedded in Epon 812, and semi-thin (1 µm) tissue sections were stained with toluidine blue. Areas containing RCMV-infected cells were selected using light microscopy. Selected areas were processed for ultra-thin sectioning (70 nm), and ultra-thin sections were contrasted according to Reynolds, using 2% uranyl acetate (15 min) and lead citrate (2 min). Finally, transmission electron microscopy (TEM) analysis was performed at 60 kV using a transmission electron microscope (Philips, EM201, The Netherlands).

Assessment of infectious virus

The amount of infectious virus was determined in salivary glands using a plaque assay with rat embryonic fibroblasts (REFs) as described previously [11]. In short, the organs were homogenised and suspended in minimal essential medium containing 2% fetal calf serum. Tenfold dilutions of 10% homogenates (w/v) were inoculated on confluent REF monolayers. After an incubation period of 7 days under 0.25% agarose, the number of plaques was determined microscopically after fixation and methylene blue staining.

Statistics

For statistical analysis, the two-tailed, non-parametric Mann Whitney U test was used. Differences associated with $P<0.05$ were considered statistically significant. Data were presented as mean±SEM.

Results

Localisation of RCMV infection in the salivary glands of young adult rats

Infection of salivary glands was studied in young adult rats using several virological and histological techniques at 7, 14 and 28 days p.i. The presence of infectious virus in submandibular, parotid and sublingual glands was tested using a plaque assay at 14 days and 28 days p.i. The submandibular and parotid gland yielded high concentrations of infectious virus: 8.09±0.25 and 4.98±0.22 ^{10}log PFU per gram tissue at 14 days p.i., respectively (Table 1). Production of infectious virus increased in these organs during the course of infection. At 28 days p.i., production was 10.90±0.17 ^{10}log PFU for the submandibular and 8.54±0.40 ^{10}log PFU for the parotid gland. In contrast, the amount of infectious virus found in the sublingual gland decreased from 8.51±0.28 ^{10}log PFU at 14 days to 6.91±0.67 ^{10}log PFU at 28 days p.i. The number of infected cells within these glands was determined using the ISH technique and is expressed as the number of positive cells per 1000 striated duct cells, as shown in Table 1. The number of infected cells during the course of infection correlated accurately with the data from the plaque assays. Data from both techniques indicate that at 28 days p.i., the submandibular gland is the preferred organ for RCMV replication, while the parotid and the sublingual glands contained significantly lower concentrations of virus.

Immunohistochemistry and ISH revealed that infection was confined to striated duct cells in submandibular, parotid and sublingual glands of immunosuppressed young adult animals. Granular convoluted duct cells, acinar cells, intercalated duct cells and interlobular duct cells remained negative during the course of infection. Infection of immunocompetent animals resulted in the same viral tropism for striated duct cells, although the concentration of infectious virus and virus-infected cells was lower or even undetectable (data not shown).

Uninfected striated duct cells showed a rectangular shape (17.22±0.13×9.21±0.45 µm) with their small basis attached to the basal membrane. The cells contained a centrally located round nucleus (7.40±0.13×6.55±0.14 µm). At the basis of the cell, multiple invaginations could be appreciated. The most prominent finding in RCMV-infected striated duct cells was the enormous increase in total cell size ($P<0.05$ when compared with non-infected; Fig. 1A). The cytoplasm protruded into the lumen of the duct and, in some cases, multiple infected cells resulted in a partial or even complete obliteration of the duct. The cytoplasm was filled with granular material, which gave the cells a foam-like appearance. This increase in cytoplasmic volume resulted in a more basally located nucleus and disappearance of the invaginations. In contrast to non-infected striated duct cells, the nucleus had an oval shape (16.04±0.64×8.96±0.55 µm) and was significantly enlarged (Fig. 1A). In the duct, infected cells were most frequently lined by non-infected cells (Fig. 2A), and infection was focally distributed throughout the gland.

In contrast to the submandibular gland, the parotid gland was characterised by a more lobular structure of the gland and the absence of granular convoluted duct cells. The sublingual gland also lacks this duct segment. In both glands, infection was also confined to striated

Table 1 Presence of rat cytomegalovirus (RCMV) in the salivary glands of 6-week-old rats. The rats received 5 Gy total body irradiation (TBI) before intraperitoneal (i.p.) inoculation with 10^5 plaque-forming units (PFU) RCMV at the age of 6 weeks. *ND* not determined; *p.i.* post infection

Salivary glands	Days p.i.	RCMV-positive cells[a]	Viral load[b]
Submandibular gland	7	0±0	ND
	14	114±18	8.09±0.25
	28	116±19	10.90±0.17
Protid gland	7	0±0	ND
	14	10±4[c]	4.98±0.22[c]
	28	55±24[c]	8.54±0.40[c]
Sublingual gland	7	0±0	ND
	14	95±35[c]	8.51±0.28
	28	53±21[c]	6.91±0.67[c]

[a] RCMV-infected cells were detected using in situ hybridisation (ISH) and the number of positive cells is expressed per 1000 striated duct cells (mean±SEM)
[b] Viral load was measured using plaque assay and expressed as ^{10}log PFU/g tissue (mean±SEM)
[c] Significant ($P<0.05$) when compared with the submandibular gland

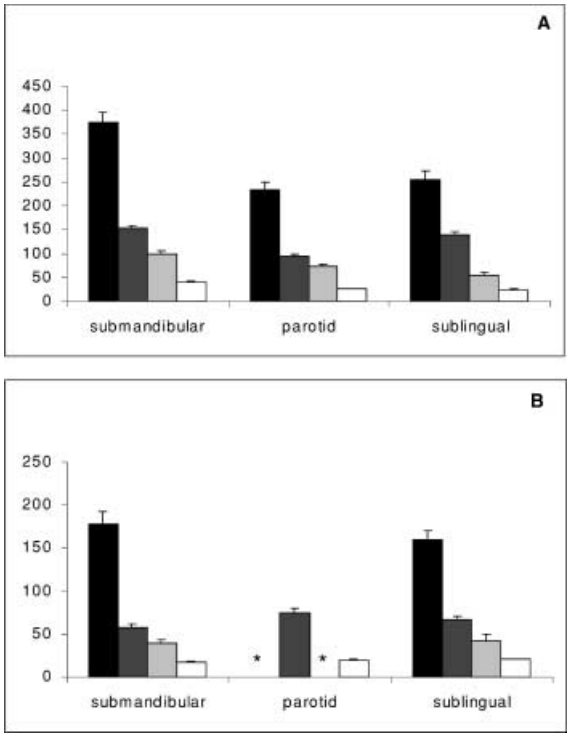

Fig. 1 Area of rat cytomegalovirus (RCMV)-infected [measured using in situ hybridisation (ISH)] versus non-infected striated duct cells in the submandibular, parotid and sublingual salivary glands extracted from young adult (A) and new-born (B) rats. *Black bar* total cell area of RCMV-infected striated duct cells; *dark grey bar* nuclear area of RCMV-infected striated duct cells; *light grey bar* total cell area of non-infected striated duct cells; and *white bar* nuclear area of non-infected striated duct cells. Data are expressed as mean±SEM (μm^2). All differences between infected and non-infected cells are significant ($P<0.05$). Using ISH, no viral DNA could be detected in parotid glands (*) extracted from new-born rats

duct cells. There were no differences in morphology of infected cells among the submandibular, parotid and sublingual glands.

In addition to light microscopy, electron microscopy was used as a golden standard for identification of infected cells in the submandibular, parotid and sublingual glands. The observations found using light microscopy were confirmed using EM. Infected cells were characterised by an enlarged oval-shaped nucleus and with an increase in total cell size, as described above (Fig. 1B). The surrounding cells were characterised by basal invaginations, the presence of many mitochondria and a centrally located nucleus indicating that the infected cells are part of the striated duct (Fig. 2A). In the nucleus of infected cells, many immature virus particles could be visualised.

Figure 2B suggests the budding of a mature virus particle from the nuclear membrane (arrowhead 8). Additionally, several relatively small vesicles containing mature and immature viral particles and dense bodies assemble in the cytoplasm. As depicted in Fig. 2D, the mature virus particles consist of a nucleocapsid with an electron-dense DNA structure. The nucleocapsid is surrounded by tegument and viral envelope. The large vesicle fuses with the cellular membrane and releases its contents into the lumen (Fig. 2C). In conclusion, RCMV replication in salivary glands of young adult Lewis rats is exclusively confined to striated duct cells. This was shown using immunohistochemistry, ISH and EM techniques.

Table 2 Presence of rat cytomegalovirus (RCMV) in the salivary glands of new-born rats. Rats were infected with 10^4 plaque-forming units (PFU) RCMV at day 2 after birth and were sacrificed at day 21 post infection (p.i.)

Salivary glands	RCMV-positive cells[a]	Viral load[b]
Submandibular gland	23±8	5.6±0.5
Parotid gland	0±0	3.1±0.4
Sublingual gland	66±17[c]	7.0±0.3[c]

[a] Measured using in situ hybridisation (ISH) and expressed per 1000 striated duct cells. The results are expressed as mean±SEM
[b] Viral load was measured using a plaque assay and expressed as ^{10}log PFU/g tissue (mean±SEM)
[c] Significant when compared with the submandibular gland ($P<0.05$)

Localisation of RCMV in the salivary glands of the new-born rat

Preliminary data in our laboratory suggested that infected cells were of granular convoluted duct origin due to the characteristic appearance of infected cells. Data from young adult animals shown above suggested otherwise. To confirm our data from the young adult animals, a second model was employed that lacked the putative target cells. This model consisted of infection of new-born rats at the age of 3 days. The animals were sacrificed 21 days p.i., i.e. just before onset of granular duct development. As shown in Table 2, infectious virus could be detected in the salivary glands of these animals, especially in the submandibular and sublingual glands. In contrast to young adult animals, the amount of infectious virus and the number of RCMV-infected cells in new-born animals were significantly higher in the sublingual gland than in the submandibular glands. Low concentrations of infectious virus, measured using a plaque assay and no viral DNA using ISH, were found in the parotid glands of these animals. In concert with their young adult counterparts, viral replication was restricted to striated duct cells and similar cytopathology of the infected cells was found (Fig. 1B). In conclusion, these experiments in new-born rats showed that, despite differences in salivary gland tropism, RCMV replication in these animals is also confined to striated duct cells.

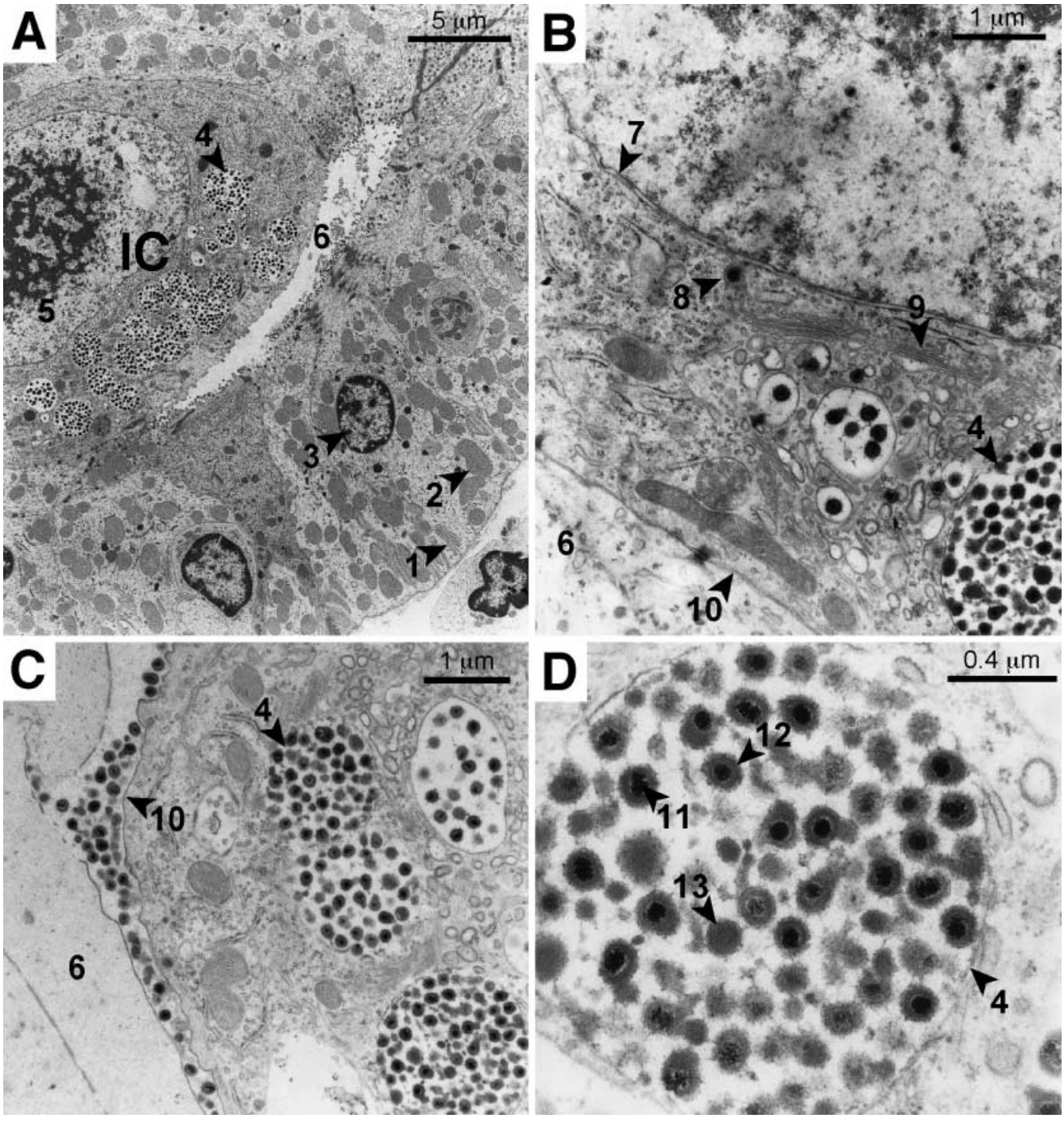

Fig. 2 Striated duct cells are characterised by basal striations, a centrally located nucleus and many mitochondria. The infected cell (IC) is located within the striated duct (**A**). Assembly of small vesicles containing mature and immature viral particles at the Golgi apparatus (**B**). The nucleocapsid is surrounded by tegument and viral envelope. Release of virus particles in the lumen of the duct (**C**). The mature virus particles consist of a nucleocapsid with an electron dense DNA structure (**D**). *Arrowheads* indicate the following structures: *1* basal striations; *2* mitochondrium; *3* uninfected nucleus; *4* virus-containing vesicle; *5* rat cytomegalovirus (RCMV)-infected nucleus; *6* lumen; *7* nuclear membrane; *8* the budding of a virus particle from the nuclear membrane; *9* Golgi apparatus; *10* cellular membrane; *11* nucleocapsid; *12* tegument and viral membrane; and *13* dense body

Inflammatory cell influx in the RCMV-infected salivary glands

RCMV infection of the salivary glands of young adult rats generated a cellular inflammatory response as shown by the presence of monocytes/macrophages, lymphocytes and NK cells at day 14 and day 28 p.i. (Table 3). At these time points, the number of inflammatory cells in the submandibular and in the parotid glands was significantly higher than in the glands of mock-infected animals. In the sublingual glands of the infected rats, no increased influx of inflammatory cells was observed com-

Table 3 Presence of monocytes/macrophages, T lymphocytes and natural killer (NK) cells in the salivary glands of rat cytomegalovirus (RCMV)-infected rats. Rats received a total body irradiation of 5 Gy before inoculation with 10^5 plaque-forming units (PFU) RCMV. The animals were sacrificed at different time points and the salivary glands were used for this study. The presence of cells was assessed semi-quantitatively. Absence of cells –; sporadic cells ±; small numbers of cells +; considerable number of cells ++; high numbers of cells +++

Glands	Infection	Monocytes/macrophages[a]			T lymphocytes[b]			NK cells[c]		
		7	14	28	7	14	28	7	14	28
Submandibular	RCMV	±	+++	+++	±	+	+++	±	+/++	+
	Mock	±	+	+	±	±	±	±	±	±
Parotid	RCMV	±	+++	++/+++	±	+	++/+++	±	+/++	±
	Mock	±	+	+	±	±	±	±	±	±
Sublingual	RCMV	±	±	±	±	±	±	±	±	±
	Mock	±	±	±	±	±	±	±	±	±

[a] Cells reactive with monoclonal antibody (mAb) ED-1, specific for monocytes/macrophages
[b] Cells reactive with mAb W3/13, specific for T lymphocytes
[c] Cells reactive with mAb 3.2.3, specific for NK cells

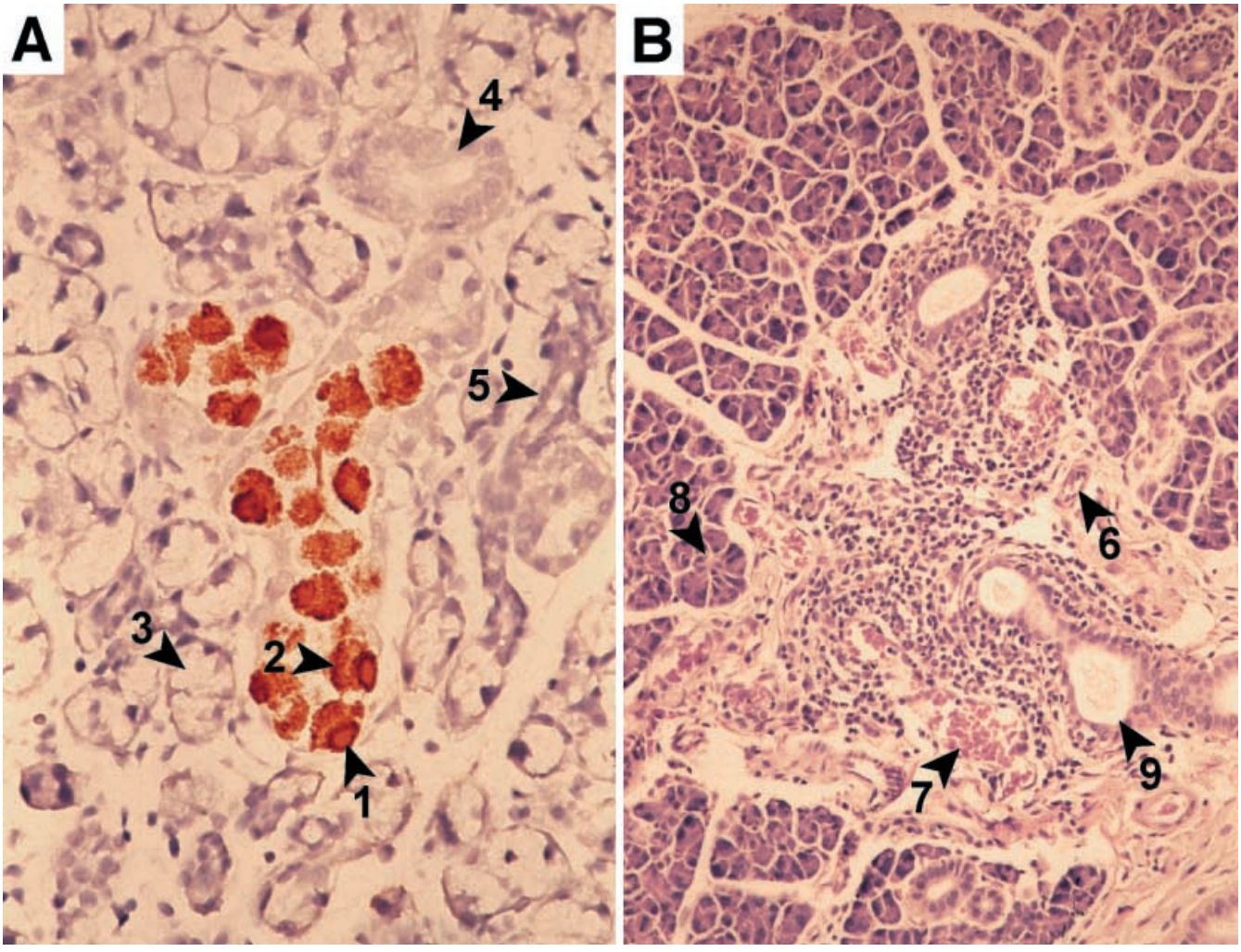

Fig. 3 **A** Detection of viral DNA using in situ hybridisation (ISH) in the submandibular gland of a young adult rat at 28 days post infection (p.i.). The nuclear staining of rat cytomegalovirus (RCMV)-infected cells (*arrowhead 1*) can be distinguished from the cytoplasmic staining of virus-containing vesicles (*arrowhead 2*). Note the relative absence of inflammatory cells. **B** Parotid gland of a young adult rat at 28 days p.i. Note that the influx of inflammatory cells is predominantly confined to the interlobular duct region. *Arrowheads* indicate the following structures: *3* acinus; *4* uninfected striated duct segment; *5* intercalated duct segment; *6* artery; *7* vein; *8* acinus; and *9* interlobular duct

pared with controls. The influx of monocytes/macrophages preceded the influx of T lymphocytes in the submandibular and the parotid glands. A similar observation was reported previously by Persoons et al. [38] using the subcutaneously infected hind-paw model.

The influx of NK cells was rather low and was maximal at 2 weeks p.i. (Table 3). This is of importance since it has been suggested that NK cells in particular are responsible for the clearance of CMV infection [6, 51]. Another interesting finding in this study was that inflammatory cells detected in the infected glands were not localised around the infected striated duct cells, as expected (Fig. 3A), but were confined to the interlobular duct region in the submandibular and parotid glands (Fig. 3B). This was true for both immunocompetent and immunosuppressed animals (data not shown). Only a few inflammatory cells were present in the sublingual gland (Table 3). This is an intriguing observation since it could be one of the mechanisms involved in the persistence of infected cells in this organ.

Discussion

In this study, a detailed description of CMV replication in the submandibular, parotid and sublingual salivary glands of the rat is reported. The main objective of the present study was to assess the identity of the type of RCMV-infected cells in the organs. Initially, preliminary data suggested that infected cells were of granular convoluted duct origin due to the characteristic appearance of the infected cells. Through the use of different techniques (immunohistochemistry, ISH and EM), it became clear that not the granular convoluted duct cells but the striated duct cells were infected. This finding was further supported by the results obtained in i.p.-infected newborn rats that were sacrificed before onset of granular duct development (at 4 weeks of age). Our study clearly shows that RCMV replication was exclusively confined to striated duct cells in all three salivary glands and that acinar cells, intercalated duct cells, granular convoluted duct cells and the interlobular duct cells remained free of virus. This is of interest, since striated duct cells differentiate into granular convoluted duct cells starting at 4 weeks of age [30] and, as a result, the ability for RCMV to replicate in these cells is lost in the process. Restriction of CMV replication to (a segment of) ductal cells in the rat model is in concert with the data obtained in the guinea-pig infection model using guinea-pig CMV (GPCMV), in which infection was limited to ductal segments [5, 22, 23, 25]. In humans, CMV-infected ductal cells and acinar cells were found [24, 39, 45, 50]. In mice, only infected acinar cells were described [26, 27, 28, 29, 31, 34, 36]. No clear explanation for these differences in infection of salivary glands between different models has been given. The difference between RCMV and MCMV in their behaviour for salivary gland cell tropism is especially fascinating since it is known that these viruses possess a lot of similarities and the genomes of both viruses show the highest homology of all known CMVs [1, 2, 3, 4]. Another interesting observation of this study is that, although RCMV replicates only in the striated duct cells in the salivary glands, the virus content is significantly different among the different glands, suggesting that other yet unexplained factors are also involved in the infection and production of virus in these glands.

An important characteristic of CMV infection is its persistence in salivary glands. In the rat model, persistent infection accompanied by continuous shedding of virus from this organ was shown previously [13]. The cells responsible for this persistence and shedding are striated duct cells, since only these cells contain high amounts of virus particles, as shown using immunohistochemistry, ISH and EM. From our in vivo data and from results obtained with other cells, such as endothelial and smooth muscle cells, we know that at least two factors contribute to persistent infection. These factors include the type of cell that is infected and the immune response of the host to this infection. Depending on the type of cell that is infected, an infection results in cell death (fibroblasts [11]), an abortive infection (endothelial cells [9]) or a persistent infection (smooth muscle cells and monocytes/macrophages [48, 21])

In the present study, we detected not only that striated duct cells are the preferred cells for CMV infection in the salivary glands but also that in these cells the production of virus particles follows a different course than in fibroblasts. While in fibroblasts, like in neo-intimal smooth muscle cells, high concentrations of virus particles were found in the nucleus [37, 47], we detected large amounts of virions in vacuoles in the cytoplasm of striated duct cells. Such observations have thus far not been performed in other cell types of rat origin. It suggests that replication, production and excretion of the virus in striated duct cells is different from replication in fibroblasts and is cell-type dependent. However, it should be noted that virus-containing vesicles are a common observation in the human system [35].

In vivo, the situation is even more complex; the cell type is important, but other factors such as the immune response of the host are also significant. In our study, it was found that, in the infected salivary glands, inflammatory cells were not localised directly around the infected cells but were localised at the interlobular duct regions. This is an interesting finding since it might explain the persistence of CMV in salivary glands. A similar observation has also been described for the mouse model [26], indicating that, in contrast to other CMV infected organs, the inflammatory cells in the salivary glands do not reach the infected cells. Thus they are not able eliminate virus-infected cells and by this way limit the infection. The reason for this remains unknown.

However, it is known that, eventually, the virus is removed from the gland [12]. In addition, EM showed that viral excretion from the infected cell occurs by apical cell budding of the larger virus-containing vesicles. It should be noted, however, that transmission of virus to

other cells via direct cell–cell contact could not be excluded using EM. A possible mechanism for viral clearance from these organs might be that infected cells can only infect downstream-located striated duct cells. During the course of infection, infected cells located in the proximal striated duct segment would die and would be replaced by non-infected counterparts. As a result, the infection would be removed from the gland in the direction of saliva flow without intervention of the immune system. Adding to this is the fact that the number of striated duct cells decreases with ageing of the rat due to the differentiation of striated duct cells into granular convoluted duct cells [30].

Also, other mechanisms could be involved in the persistence of the infection in the salivary glands; for example the observation that, in contrast to other organs, the NK cell influx in this organ is only slightly enhanced, while in other infected organs a high influx of NK cells is found [49]. Since it is known that these cells are important cells for the clearance of CMV-infected cells [44], diminished influx of NK cells could play a role in persistence of the virus in the salivary glands.

In conclusion, we showed that RCMV exclusively infects striated duct cells in the salivary glands. In these cells, high levels of infectious virus are produced during a long period p.i. Persistence in the salivary glands is partly due to the type of cell that is infected but probably also to a failure of the immune reaction in removing the virus-infected cells in these organs. The difference between MCMV and RCMV in preference of infection site is an interesting observation that cannot yet be explained. The use of in vitro cell culture in the near future will give us more answers about infection and persistence of CMV in salivary glands.

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