SITE-RESTRICTED PERSISTENT CYTOMEGALOVIRUS INFECTION AFTER SELECTIVE LONG-TERM DEPLETION OF CD4⁺ T LYMPHOCYTES

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Cytomegalovirus (CMV) infections are controlled by the immune system and lead to viral latency (1) in the immunocompetent host. Only in the immunocompromised host do primary as well as recurrent infections cause fatal disease. Congenital infection is responsible for severe fetal malformation (2, 3), and interstitial CMV pneumonia is considered the main reason for mortality in leukemia patients irradiated for bone marrow transplantation (4). Manifestations of CMV disease have been reported as episymptoms of AIDS, frequently representing the immediate cause of death after immunosuppression by HIV (5).

We have previously described an animal model for the study of CMV disease in the whole-body γ-irradiated host, the infection of mice with murine CMV (MCMV) (1). By adoptive cell transfer, protection against acute MCMV disease proved a function of specifically sensitized effector T lymphocytes of the CD8 subset, whereas T lymphocytes of the CD4 subset were dispensable for short-term immunocytotherapy (6–9). The CD8⁺ effector cells prevented the lethal bone marrow aplasia caused by a virus-induced failure in hemopoietic stem cell generation (10), and also prevented tissue lesions by controlling the spread of virus (8). The immunodominant antigen recognized by protective, antiviral CD8⁺ T lymphocytes could be identified as a family of peptides derived from pp89, the major immediate-early phase protein encoded by MCMV (11–17).

The conditions for superimposed CMV disease during HIV infection differ from those in bone marrow transplant recipients in that the immunosuppression selectively affects the CD4⁺ T lymphocytes, while sparing the CD8⁺ T lymphocytes (18).

It was therefore of interest to explore to what extent the residual CD8 subset can control CMV infection on its own. Recent reports have given examples for an autonomy in vivo of the CD8 subset in viral infection models involving a depletion of the CD4 subset (19–22), which implies that cooperation of CD4⁺ helper T lymphocytes is not essential for initiating a CD8 subset-mediated immune response. Whether such a response can last long enough to also maintain a long-term control

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Abbreviations used in this paper: MCMV, murine CMV; PFU, plaque-forming units.
of a persistent or latent CMV infection, was, however, still open to question. In this communication, as a model for CMV disease in the CD4 subset-deficient host, the course of MCMV infection was studied in mice that were long-term depleted of the CD4 subset with anti-CD4 mAb administered repeatedly. We report that, even though the clearance of replicating virus in host tissues was delayed, protective CD8+ effector cells were generated and finally restricted persistent virus multiplication to acinar glandular epithelial cells of the salivary glands.

Materials and Methods

Elimination of T Lymphocyte Subsets In Vivo. 8-wk-old female BALB/c mice, bred under specific pathogen-free conditions, were depleted of T lymphocyte subsets according to the protocol established by Cobbold et al. (23). Long-term depletion of the CD4 subset was accomplished by weekly intravenous infusions (for time schedule see Fig. 1) of ~1 mg of rat IgG2b antimurine CD4 mAb GK1.5 (24, 25) dissolved in 0.5 ml of PBS, and the CD8 subset was depleted by a single infusion of the same amount of rat IgG2b antimurine CD8 mAb YTS-169.4 (23). The antibodies were partially purified by precipitation with 50% ammonium sulfate from the ascites of BALB/c mice bearing the respective hybridomas. After dialysis against PBS, the amount of specific antibody in the ascites protein was determined by a radial immunodiffusion assay using rat IgG standards (Serotec, Oxxon, England).

Virus and Infection Conditions. Sucrose gradient-purified, mouse embryo fibroblast culture-propagated MCMV (Smith strain, code VR 194; American Type Culture Collection, Rockville, MD) was used for intraplantar infection. The dose of infection was 2 x 10^5 plaque-forming units (PFU) throughout. CD4 subset-depleted mice were infected at the day after the first infusion of antibody, and recipients for the adoptive transfer were infected ~2 h after γ irradiation and cell transfer.

Adoptive T Lymphocyte Transfer. A prophylactic adoptive transfer was performed as described previously (6-9). In brief, recipient mice were immunodepleted by total-body γ irradiation with 6 Gy delivered as a single dose at a rate of 1.325 Gy/min. Nylon wool-purified spleen T lymphocytes or subsets thereof, derived by in vitro depletion with antibody (GK1.5 and YTS-169.4 for depleting the CD4 and CD8 subset, respectively) and rabbit complement, were infused into the tail vein ~4 h after irradiation and 2 h before infection. Virus titers in tissues of the recipients were determined 2 wk later.

Virus Titers in Tissues. Infectious MCMV in tissues was quantitated by a plaque assay (6). The detection limit was 100 PFU of MCMV per organ homogenate. The pooled parotid, greater sublingual, and mandibular glands are collectively referred to as salivary glands. Sets of virus titers are regarded as significantly different for p < α = 0.05 (one sided), where P denotes the observed probability value, and α denotes the selected significance level (distribution-free Wilcoxon-Mann-Whitney exact rank sum test).

Detection of Virus-specific Antibodies. The ELISA for quantitating MCMV-specific antibodies in sera by using ultrasonicated, infected mouse embryo fibroblasts as source of antigen has been described in detail previously (16). Titers were read from the half-maximal OD after subtraction of values obtained when, for control, the lysate of noninfected fibroblasts was used for coating the microdilution plates. The specificity of the sera was independently controlled by the immunoprecipitation of MCMV-specific proteins (12).

Cytofluorometric Analysis. For single-parameter cytofluorometric analysis (FACS IV; Becton Dickinson & Co., Sunnyvale, CA) of the distribution of the CD4 and CD8 subsets, T lymphocytes were stained indirectly with mAb GK1.5 and YTS-169.4, respectively, using fluorescein (FITC)-conjugated affinity-purified F(ab') fragments of goat anti-rat IgG (Jackson Immuno Research, Avondale, PA) as second reagent. The fluorescence signal was triggered on particles larger than platelets, and propidium iodide (2 μg/ml) -stained dead cells were excluded by electronic gating. Relative fluorescence intensities were expressed on a log scale, with 10^4 cells analyzed.

Histology. For light microscopy, mandibular glands were fixed with Bouin's fixative. Tissue samples were embedded in paraffin, and thin sections (5 μm) were stained with hematoxylin
and eosin. For EM, fixation was performed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). After postfixation in 1% osmium tetroxide, tissue blocks were embedded in Araldite™. Semithin sections (1 μm) were stained with toluidine blue to select sites of interest by light microscopy. Ultrathin sections (70 nm) from selected sites were stained with uranyl acetate and lead citrate, and were examined with an electron microscope (model 109; Carl Zeiss, Inc., Thornwood, NY).

Results

Experimental Design. The time schedule for the treatments and assays is outlined in Fig. 1 to explain the experimental concept. It was the intention to study the autonomous contribution of the CD8 subset of T lymphocytes to the long-term control of a CMV infection. The problem falls into two main questions: first, can antiviral effector cells be generated from their precursors in the absence of CD4+ helper T lymphocytes; and second, if so, is this response lasting and can it maintain a long-term control of a persistent infection in the absence of antiviral antibody?

To address both questions in the same experiment, BALB/c mice were already depleted of the CD4 subset before the infection, and the state of selective CD4 subset deficiency was then maintained throughout the observation period of 10 wk. The efficacy of depletion was controlled quantitatively by cytofluorometric analysis of the distribution of T lymphocyte subpopulations in lymphoid tissues. One has to bear in mind, however, that cytofluorography can give us only snapshots of the composition of the T lymphocyte population and only at sites selected for testing. Since in vivo depletion of the CD4 subset has been shown to prevent the generation of antibodies (19–23, 25, 26), monitoring of seroconversion provides a sensitive and location-independent means for controlling an enduring functional depletion of helper T lymphocytes. Both approaches in combination should thus give reliable information on the completeness of depletion.

The generation of antiviral effector cells in infected, CD4 subset-depleted, as well as nondepleted, mice was tested by adoptive transfer of T lymphocytes from donors of the respective groups into syngeneic recipient mice that were immunodepleted.

![Figure 1. Experimental regimen and time schedule.](image-url)
by γ irradiation. This regimen has been worked out previously to a model for cytoimmunotherapy of CMV disease (6-10). The control of viral spread in tissues of the recipients proved a function of the number of transferred CD8+ antiviral effector cells and was neither dependent upon nor influenced by a cotransfer of CD4+ T lymphocytes (6-10). Adoptive transfer can therefore also be used as an in vivo assay for estimating the strength of the CD8 subset-mediated antiviral immunity established in the donor mice.

The state of MCMV disease in CD4 subset-depleted mice and, for control, in nondepleted mice, was followed by screening virus production in tissues of individual probands collected randomly from the respective sample.

Induction of a CD4-CD8- Subset by In Vivo Depletion of the CD4 Subset. In accord with the description of the in vivo depletion method for T lymphocyte subsets by Cobbold et al. (23) and Wofsy et al. (25), intravenous infusion of the rat antimurine CD4 mAb GK1.5 caused a rapid and complete depletion of CD4+ T lymphocytes in lymph nodes (not shown) and spleen (Fig. 2, top and center), so that at the time of infection (Fig. 1), the T lymphocyte population consisted entirely of CD8+ T lymphocytes (Fig. 2, center). To maintain the state of CD4 subset deficiency, the depleting antibody was administered weekly. When after 6 wk the composition of nylon wool-purified splenic T lymphocytes was controlled again; continued absence of CD4+ T lymphocytes could be confirmed, but in addition to the expected CD8 subset, a double-negative CD4-CD8- subset was then detected (Fig. 2, bottom). Only recently has this phenomenon been described (27), and it has been overlooked in previous reports on the in vivo autonomy of the CD8 subset. In light of the presence of a population of as yet undefined function, the role of CD8+ T lymphocytes in the CD4 subset-deficient host has to be critically re-evaluated.

The Production of Antiviral Antibodies Requires Help by the CD4 Subset. Previous reports have already given examples for the prevention of antibody responses by in vivo depletion of the CD4 subset (19-23, 25, 26). This also holds true for the antibody response to MCMV (Table I). When tested 6 and 10 wk after infection, seroconversion in immunocompetent mice became evident from high MCMV-specific antibody titers, whereas mice depleted of the CD4 subset mostly remained seronegative or at best developed a marginal antibody titer. Three conclusions can be drawn: first, the in vivo B lymphocyte response to MCMV is as strictly dependent upon the cooperation with T lymphocytes of the CD4 subset as has been seen with other antigens. Second, the in vivo depletion of the CD4 subset in our experiments was complete and lasting enough for long-term prevention of an antibody response. Third, the observed failure in antibody production implies that the newly arising CD4-CD8- lymphocytes do not substitute for CD4+CD8- T lymphocytes in providing help to B lymphocytes.

Presence of Antiviral CD8+ Effector Cells in Long-term CD4 Subset-depleted Mice. Lymphocyte transfer into lethally infected recipient mice was used as an in vivo assay for testing the presence and efficacy of protective, antiviral effector cells within the CD8 subset of T lymphocytes from donors 6 wk after infection. Splenic T lymphocytes were isolated by nylon wool purification and were depleted in vitro of the CD4 subset to make also formally sure that the assay was not influenced by CD4+ helper T lymphocytes. Virus replication in tissues of the recipients is shown representatively for the lungs as indicator site (Fig. 3), with similar data determined for spleen and adrenal glands (not shown).
**T lymphocyte subpopulations**

Immunocompetent

Short-term CD4-subset depleted

Long-term CD4-subset depleted

**Figure 2.** T lymphocyte subpopulations after in vivo depletion of the CD4 subset. Cytfluorometric analysis comparing the distribution of T lymphocyte subpopulations in immunocompetent mice (upper row), in mice 1 d after infusion of αCD4 mAb (center row), and in mice after weekly infusions of αCD4 mAb for a period of 6 wk (lower row). Fluorescence profiles seen after staining of the CD8 subset (left column) and of the CD4 subset (right column) are depicted with solid lines. The control profiles obtained with the FITC-conjugated second reagent alone are shown superimposed with dashed contours.

**Table I**

Absence of MCMV-specific Antibody in CD4 Subset-depleted, Persistently Infected Mice

<table>
<thead>
<tr>
<th>Weeks postinfection</th>
<th>αCD4 in vivo</th>
<th>Proportion of seropositive mice*</th>
<th>Antibody titer in seropositive mice†</th>
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<tbody>
<tr>
<td>6</td>
<td></td>
<td>4/4</td>
<td>2,560-5,120</td>
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<tr>
<td>6</td>
<td>+</td>
<td>2/9</td>
<td>&lt;10</td>
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<tr>
<td>10</td>
<td>–</td>
<td>6/6</td>
<td>2,560-10,240</td>
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<tr>
<td>10</td>
<td>+</td>
<td>1/5</td>
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* Serum was taken from individual mice for which the virus titers in tissues were determined at 6 wk postinfection (see Fig. 3) and at 10 wk postinfection (see Table II), respectively.

† MCMV-specific antibodies were determined by ELISA. Data represent the reciprocal of the serum dilution that gave half-maximal activity.
The difference between the virus titers seen after transfer of nonprimed CD8⁺ T lymphocytes isolated from noninfected, immunocompetent donors (Fig. 3, A) and those seen with infected, immunocompetent mice as donors (Fig. 3, B), is a measure of the antiviral response of the CD8 subset in the presence of the CD4 subset (compare A and B; p = 0.005). In the absence of the CD4 subset (Fig. 3, C) the antiviral response of the remaining T lymphocytes reached the same strength (compare B and C; p > 0.1). Since, however, after long-term depletion of the CD4 subset, the lymphocyte population was composed of a CD8 subset and of a new subset with the phenotype CD4⁻CD8⁻ (recall Fig. 2), it became necessary to also consider the possibility that the antiviral function was performed by this new subset and not, as one would anticipate from published experience (19-22), by CD8⁺ effector cells generated independently of the CD4 subset. Elimination in vitro of the CD8 subset and transfer of the resulting purified CD4⁻CD8⁻ population (Fig. 3 D) confirmed that, also in a state of CD4 subset deficiency, all antiviral effector cells belong to the CD8 subset (Fig. 3, compare C and D; p = 0.005).

Two conclusions can be drawn: first, an antiviral immune response by the CD8 subset can be initiated and maintained without the help provided by the CD4 subset; and second, the CD4⁻CD8⁻ subset has no antiviral function.

**Delayed Clearance of Infectious Virus in Tissues of CD4 Subset-depleted Mice.** The state of disease in CD4 subset-depleted mice and in immunocompetent mice was compared by quantitating virus in tissues at 6 wk after the infection (Fig. 4). In immunocompetent mice, virus spread was controlled by that time in lungs and spleen, and in the salivary glands, which are known as a preferred site of CMV replication (6-8, 28); virus was detected in only two of four mice, and then only with low titers. In the spleen of CD4 subset-depleted mice, which is at the site at which presence
of antiviral CD8+ effector cells could be demonstrated (recall Fig. 3), in six of nine mice, virus multiplication was also prevented, whereas in lungs and in salivary glands, the production of infectious virus continued. The wide range of virus titers seen for the lungs suggested that in this tissue, antiviral effector cells were operating with different efficacy among individual mice. The prolonged phase of virus replication was not associated with any sign of disease. One could think that the absence of antiviral antibody in CD4 subset-depleted mice (Table I) was responsible for enduring virus production in lungs and salivary glands. It is therefore worth recalling that strikingly similar observations were made 2 mo after successful cytotoxic immunotherapy of lethal MCMV disease in γ-irradiated mice, even though in that situation all mice were seropositive as a result of autoreconstitution (8).

**Cell Type-restricted Persistent Infection in Salivary Gland Tissue.** 1 mo later, by 10 wk after the infection, virus replication had ceased in the lungs not only of immunocompetent mice (Table II, group A), but also of CD4 subset-depleted mice, whereas in
Figure 5. Histology of a persistently infected salivary gland. Mandibular gland specimens were taken at 10 wk postinfection, which is during the period of persistent infection, from CD4 subset-depleted mice. (a) Examination by light microscopy (bar; 20 μm): (A) acinus; (D) intercalated duct in longitudinal section. A glandular epithelial cell with an intranuclear inclusion characteristic of CMV infection is seen located in one of the acini (arrow). The surrounding tissue does not show histopathologic changes. (b and c) Examination by EM (bars, 2 μm and 200 nm, respectively): (b) infected acinar glandular epithelial cell with an intranuclear inclusion body composed of reticularly arranged amorphous material surrounded by nucleocapsids. Large cytoplasmic vacuoles, oriented towards excretion ducts, are filled with high numbers (~1,000) of virions. Inset (c), content of a cytoplasmic vacuole at high magnification, demonstrating the presence exclusively of monocapsid virions.
the salivary glands virus production continued without decline (Table II, group B). In face of a permanent risk of re-infection by hematogenous spread of salivary gland-derived virus, it is remarkable that the lungs remained free of virus in spite of the absence of antibody (recall Table I). It was therefore asked whether the CD8 subset was required for the site restriction of the infection. Depletion of the CD8 subset in the long-term CD4 subset-depleted mice led to continued virus production in the lungs (Table II, group C), and thus proved that restriction of the infection to the salivary glands in persistently infected mice is a function of antiviral CD8+ effector cells.

We then studied the histological basis of the persistent infection in salivary gland tissue (Fig. 5). It was known before that in immunocompetent mice MCMV replicates without tissue damage in glandular epithelial cells of salivary gland tissue (29), whereas in γ-irradiated, immunodepleted mice, infection of connective tissue fibroblasts was also observed (F. Weiland, unpublished observations). As it has been described for embryo fibroblasts in vitro (30) and for interstitial lung fibroblasts (6), as well as for adrenal cortical parenchymal cells (9) in vivo, the morphogenesis of MCMV in salivary gland fibroblasts is characterized by the formation of multicapsid virions. During persistent infection in CD4 subset-deficient mice, infected fibroblasts and tissue lesions were not observed. Virus production was restricted to a low number of glandular epithelial cells (Fig. 5 a). The morphogenesis of MCMV is special in glandular epithelial cells in that high numbers exclusively of monocapsid virions are released from large cytoplasmic vacuoles (Fig. 5, b and c) (estimated 1,000 virions per vacuole, which is in the order of 10⁴/cell), which explains the high virus titers observed in salivary glands despite the low number of infected cells. From the lack of infected fibroblasts in CD4 subset-depleted mice along with the observation of an increase in virus titers after depletion of the CD8 subset (Table II; compare groups B and C, significant with \( p = 0.005 \)), we conclude that salivary gland tissue is accessible to antiviral control by CD8+ effector cells, and that persistent CMV infection of the host is based on an as yet undefined but selective immune escape mechanism characteristic of glandular epithelial cells.

Discussion

The aim of this study was to investigate whether in a state of selective CD4 subset deficiency the residual CD8 subset can control a CMV infection on its own, and how the course of infection, which is the succession of acute phase, persistent phase, and latent phase of infection, is altered in the absence of the CD4 subset.

It was known before from our own studies that the antiviral effector cells that protect against murine CMV disease belong to the CD8 subset (6-10, 16). The question to be answered was, therefore, whether the antiviral response of this subset can be initiated and also maintained long enough for controlling the infection without help delivered by the CD4 subset. We could add a new example to the increasing evidence that CD4+ helper T lymphocytes are not essential for initiating a CD8 subset-mediated response in vivo (19-22, 31, 32), and, in addition, the data affirmed that the antiviral function of the CD8 subset in CD4 subset-depleted mice is not rapidly exhausted, but is enduring. Yet, the proposed autonomy in vivo of the CD8 subset is not proven by these findings, because a CD4+CD8- subset of as yet
undefined function was seen to have accumulated in the spleen in response to the depletion of the CD4 subset.

In lymphoid tissues of immunocompetent mice, as well as of humans, CD4+CD8+ T lymphocytes are rare, but this subset was found amplified in states of defective T lymphocyte maturation or immunodeficiency (33, 34), and, specifically, an accumulation of Thy-1+CD4+CD8+ T lymphocytes in the spleen was seen also in mice depleted of the CD4 subset by infusion of αCD4 antibody (27). It is now established that T lymphocytes of this subset express a TCR-γ/δ (35–38) and are capable of performing nonrestricted (39, 40) as well as MHC-linked specific cytolytic functions (41, 42).

A role for this subset in the control of viral diseases was not yet explored. The fact that a CD4−CD8− subset accumulated after selective depletion of the CD4 subset also in the spleen of mice that were persistently infected with MCMV provided an excellent model for studying the contribution of this subset to antiviral immunity over a long period of time. Two definite conclusions can be made: first, CD4−CD8− T lymphocytes do not substitute for the depleted CD4 subset in providing help to B lymphocytes; and, second, upon adoptive transfer, CD4−CD8− T lymphocytes generated in infected mice do not function as antiviral effector cells controlling virus production in tissues. Since, however, specific in vivo depletion of this subset or prevention of its generation is technically not feasible as yet, a role for CD4−CD8− T lymphocytes in maintaining the antiviral response of the CD8 subset is not excluded, and this implies that an autonomy of the CD8 subset is not proved.

Even though virus production finally ceased in spleen and lungs under the influence of the antiviral function of CD8+ effector cells, the control of acute infection was delayed in CD4 subset-deficient mice. It could be demonstrated that at a time when the spleen was cleared in both the immunocompetent and the CD4 subset-depleted mice, the antiviral efficacy of the CD8 subset recovered from the spleen was the same in both groups of mice, as assessed by adoptive transfer. Our previous studies did not give any indication of a direct antiviral function of the CD4 subset (6–10), and since prolonged virus production in the lungs was seen also in seropositive mice (8), the absence of antibodies also does not offer the explanation for the delay in the control of infection. It remains to be tested whether a role in vivo of CD4+ helper T lymphocytes could be to accelerate the clonal expansion of the antiviral CD8+ effector T lymphocytes or to facilitate their infiltration into nonlymphoid tissue. In essence, it can be concluded that depletion of the CD4 subset affects, but does not prevent the clearance of productive acute infection in vital tissues.

While in immunocompetent mice viral latency is established, CD4 subset-deficient mice cannot eliminate virus production in the salivary glands and thus remain persistently infected. As opposed to the prolonged, but finally declining infection of the lungs, the infection of the salivary glands was truly persistent in that virus production was undiminished over an observation period of 5 mo (not shown). If we understand latency in a clinical sense as a state of disease in which virus is not replicating at any site, but can be induced to recurrence, then CD4 subset-deficient mice fail to establish latency. Yet, it would be incorrect to interpret this as a general incompetence of the CD8 subset to aid the establishment of a latent infection. There is evidence for the spleen being a site of MCMV latency (43–48); and the spleen was,
in fact, the first tissue at which productive infection discontinued under the influence of antiviral CD8⁺ effector cells.

The basis of the persistent infection in salivary gland tissue rather appears to be an as yet undefined immune escape mechanism of acinar glandular epithelial cells. In this cell type, MCMV replicates with a morphogenesis distinct from that seen for other cell types (6, 9, 29, 30), and it can be speculated that viral antigen processing and presentation may thus also differ.

In conclusion, our results in a model system for CMV disease have revealed that a selective deficiency of the CD4 subset does not prevent the control of CMV infection by antiviral effector cells of the residual CD8 subset.

Summary

We have established a murine model system for exploring the ability of a CD4 subset-deficient host to cope with cytomegalovirus infection, and reported three findings. First, an antiviral response of the CD8 subset of T lymphocytes could be not only initiated but also maintained for a long period of time despite a continued absence of the CD4 subset, whereas the production of antiviral antibody proved strictly dependent upon help provided by the CD4 subset.

Second, no function in the defense against infection could be ascribed yet to CD4⁻CD8⁺ T lymphocytes, which were seen to accumulate to a new subset as a result of depletion of the CD4 subset. This newly arising subset did not substitute for CD4⁺ T lymphocytes in providing help to B lymphocytes, and was also not effective in controlling the spread of virus in host tissues. As long as a function of these cells in the generation and maintenance of a CD8 subset-mediated response is not disproved, caution is indicated with concern to an autonomy of the CD8 subset.

Third, even though with delay, the CD8⁺ effector cells raised in the CD4 subset-deficient host were able of clear vital tissues from productive infection and to restrict asymptomatic, persistent infection to acinar glandular epithelial cells in salivary gland tissue.

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References
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