Pathogenesis of Chronic Mouse Cytomegalovirus Infection in Submaxillary Glands of C_3H Mice

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THE CYTOMEGALOVIRUSES belong to the herpes group of viruses, produce Type A nuclear inclusions, and are found in many species of animals including man. The human, primate, and murine strains cause chronic infection of the salivary glands and kidneys in the presence of circulating antibody.[1-3] In man, under conditions of severe debility or immunologic impairment, human cytomegalovirus often causes a disseminated infection which can be fatal.[1,4,5] Serologic and virologic studies in human allogenic renal transplant recipients have suggested that, during immunologic supression, cytomegalovirus infection results from either a newly acquired infection or from activation of a latent infection.[5] The mechanisms of latency and of viral activation are unknown.

In our investigations of latent cytomegalovirus infection we have used the intranuclear inclusion as a histologic marker of infected cells in order to study the reaction of the host to chronic infection. Previously, we compared the reaction of the host in organs which do and do not support chronic mouse cytomegalovirus (MCMV) infection.[6] Here, we describe the pathogenesis of chronic MCMV infection in the submaxillary glands of C_3H/Anf (mammary tumor agent free) mice and the effect of cortisone on chronic infection.

Material and Methods

Female C_3H/Anf mice, (Cumberland View Farms) 9–12 weeks of age were used in all experiments. ICR/HA female mice, 6–7 weeks of age, were also used in some experiments.

Experimental Procedure

Mice were inoculated intraperitoneally (IP) with 0.25 ml of virus suspension prepared from saline homogenates of salivary glands of ICR/HA infected mice. Virus suspensions were initially prepared as a 20% homogenate and diluted 10^{-2}

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Supported by Grant 2G-129 from NIH, US Public Health Service, and by The Otho S. A. Sprague Memorial Institute.

Accepted for publication Oct 2, 1969.

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to $10^{-3.5}$ before injection. Injections of undiluted virus suspensions regularly caused fatal disease of all mice in 4–7 days and represented LD_{100}. Periodically, after infection, 4 or 5 mice were killed and the submaxillary glands removed. The glands from 2 or 3 mice were frozen for virus assay and the glands from the other mice fixed in formalin for histology. Virus and methods of assay have been described.[6,7] Virus could not be isolated from the submaxillary glands of noninfected mice.

Two groups of mice were used in the cortisone experiments. One group consisted of control infected mice and the other of cortisone-treated infected mice. The control group received no further injections except at the time of infection.

Cortisone Administration

Cortone (Cortisone acetate—Merck, Sharpe, and Dohme, West Point, Pa) was used. Mice were injected IP 3 times every week beginning with 3.5 mg cortisone per injection and increasing the dosage 0.5 mg per injection every 2 weeks. Injections were started 7–9 days after infection with MCMV. Oxytetracycline HCl (0.33 mg/ml) was incorporated in the drinking water to minimize secondary bacterial infections.

Histology

Tissues were fixed in 10% formalin and paraffin embedded sections stained with hematoxylin and eosin. Reticulum stains were done by the Gridley method.

Results

Chronic infection in C_3H and ICR mice compared

Text-fig 1 shows virus titers in the submaxillary glands of C_3H and ICR mice during chronic infection. Peak titers occurred within 3 weeks after infection and were approximately similar for both strains, if the same dose of virus was used for inoculation. After 4 weeks, titers fell rapidly in C_3H mice, while declining more slowly in ICR mice. Once virus titers had dropped, there was no increase, at least within the time limit of the experiments. C_3H mice were used because virus titers dropped to low levels within a reasonable period of time after infection.

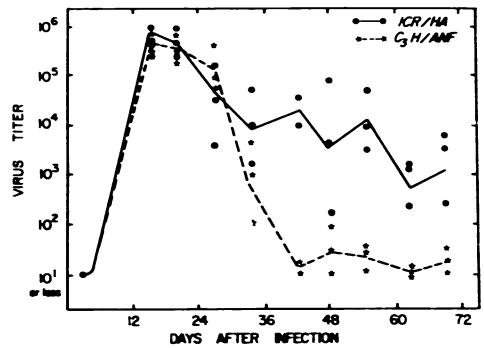
Result of reinfection

Reinfection of chronically infected C_3H mice by subcutaneous injection of LD_{100} MCMV caused no mortality or change in the levels of virus titers in the submaxillary glands when sampled on Days 9, 16, and 23 after reinfection. Even 6 months after infection, mice were resistant to reinfection with lethal doses of MCMV.

Histopathology of chronic infection in the submaxillary glands

With the appearance of inclusions by Day 7, lymphocytes and plasma cells began to infiltrate the interstitium and to surround acini in which there were infected cells. The chronic inflammatory cells continued to

accumulate and distorted and compressed the acini. Accumulation of inflammatory cells in areas appeared to be preceded by inclusions in the area (Fig 1). By 3–4 weeks, when inflammation was considered to be maximal, inflammatory cells began to permeate the acini particularly in areas of heavy infiltration, and to cause lysis and degeneration of infected cells (Fig 2). Degeneration of infected cells was most pronounced between 3 to 5 weeks after infection and was associated with decreasing virus titers in the submaxillary glands (Text-fig 1). By Day 40, intact inclusion-bearing cells could rarely be found.



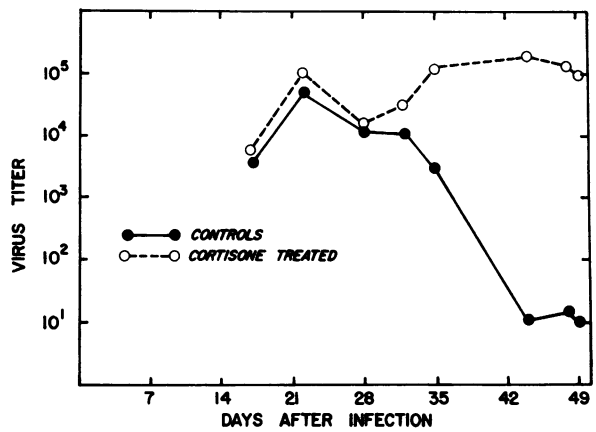
TEXT-FIG 1. Virus titers in submaxillary glands of ICR and C_3H mice during chronic infection. Both strains of mice were infected at the same time with $10^{-2.3}$ dilution salivary gland homogenate. Dots (ICR) and stars (C_3H) represent virus titers of individual animals. Virus titers are expressed as $\log_{10}$ plague-forming units in 0.2 ml/pair of salivary glands as a 10% homogenate. Lines are drawn through average values.

The inflammation was proportional to the number of inclusion-bearing cells. When low doses of virus were used, only a few inclusions appeared and the inflammatory reaction was correspondingly less, and focal. In the mucus-secreting portion of the gland, inclusions rarely occurred even with high virus doses, and here the inflammation was correspondingly mild. Inflammation did not occur in the submaxillary glands of mice injected with homogenates prepared from salivary glands of noninfected mice.

Silver impregnation stains revealed a delicate reticulum network around individual acini. With accumulation of inflammatory cells, the network often became distorted and irregular with the acini compressed. However, as long as the reticulum network remained intact, infected cells could still be recognized within the acini. With dense accumulation of inflammatory cells, 3–4 weeks after infection, there was eventual fragmentation of the reticulum fibers, collapse of the acini, and degeneration of infected cells (Fig 3).

Effect of long-term cortisone administration

Text-fig 2 shows effect of long-term cortisone administration during chronic infection. In contrast to control infected mice, virus titers in the



TEXT-FIG 2. Effect of long-term cortisone administration on virus titers in submaxillary glands of C_3H mice. Cortisone begun on Day 7 after infection and continued to end of experiment. (See *Materials and Methods* section for dosage of cortisone.) Each point represents average virus titer of 2 mice.

submaxillary glands did not decline but remained elevated at peak levels.

Histopathology of the salivary glands of cortisone-treated mice

Cortisone abolished the interstitial inflammatory reaction throughout the treatment period. Acini with infected cells remained intact and the glandular architecture was not distorted. Numerous inclusion-bearing cells persisted in the acini. Inclusions were usually very prominent and the infected cells often showed extreme cytomegalia, frequently distending the acini. There was no lysis or degeneration of infected cells that could be recognized (Fig 4). Persistence of inclusion-bearing cells correlated with elevated virus titers in cortisone-treated mice. The reticulum framework around acini was preserved, even around those containing infected cells (Fig 5).

Histologic study of mice which died or were killed for virus assay during cortisone treatment showed no evidence of visceral cytomegalovirus dissemination. Common lesions seen in cortisone-treated mice were cardiac calcification and hepatic abscesses.

Healing

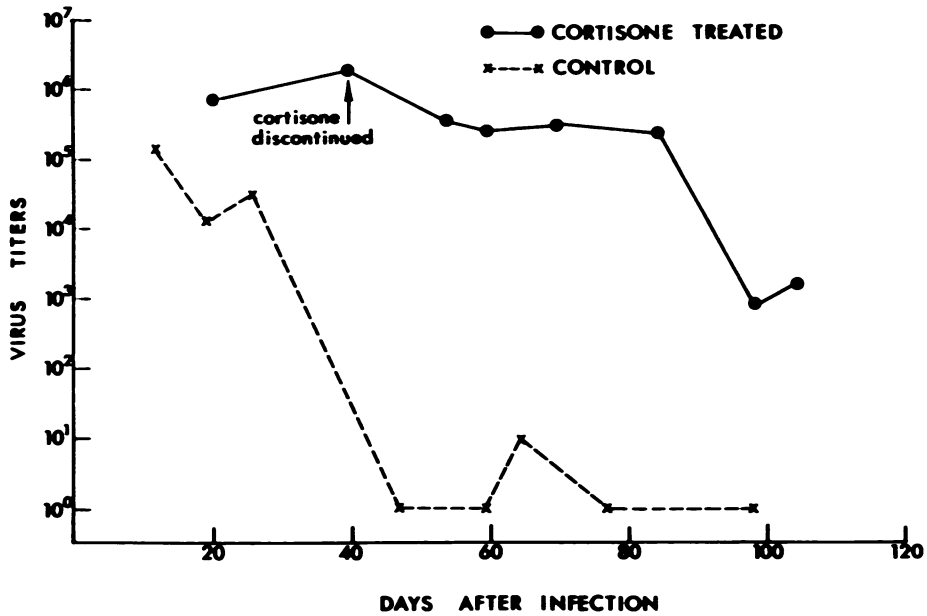
After Day 40, inclusions were rarely visible in control infected C3H mice. Inflammatory cells persisted longer, but eventually disappeared. The acini regenerated and the normal architecture was restored. No residual fibrosis or atrophy was found in the submaxillary glands.

Attempts to reactivate chronic CMV infection in the submaxillary glands

Attempts were made to reactivate cytomegalovirus in the submaxillary glands of chronically infected mice after virus titers had dropped to low levels. They included injections of adrenalin (.025 ml of 1:1000 dilution for 2 days), isoproterenol (40 μg IP for 7 days) which causes increased deoxyribonucleic acid (DNA) synthesis in the salivary glands,[8] and cortisone administration for 40 days. All attempts failed. In every experiment, there was no change in the virus titers or increase in the number of inclusions in the submaxillary glands.

Result of discontinuing cortisone

Cortisone was given from Day 7 to Day 40 after infection. Virus titers in the submaxillary glands remained elevated at peak levels during treatment and for approximately 7–8 weeks after the cortisone was discontinued, then declined (Text-fig 3). Histologic examination showed slow



TEXT-FIG 3. Result of discontinuing cortisone injection on virus titers in submaxillary glands of C3H mice. Cortisone injections were started on Day 7 and stopped on Day 40. Each point is average titer of 2 mice.

accumulation of inflammatory cells in the interstitium beginning after cortisone injections were stopped (Fig 6). Eventually, the histologic picture came to resemble that seen in control infected mice with acinar-rhexis and destruction of infected cells, which corresponded to decreasing virus titers. Failure of virus titers to drop immediately after cortisone withdrawal seemed to be due to the very slow accumulation of inflammatory cells in the submaxillary glands.

Effect of reinfection of chronically infected mice treated with cortisone

Since cortisone administration did not lead to a generalized disease in chronically infected mice, it was of interest to see if cortisone-treated mice were susceptible to reinfection with LD_{100} MCMV. Mice were started on cortisone 8 days after infection. On Day 42, they were injected with LD_{100} MCMV. The results (Table 1) showed that cortisone-treated mice are as resistant to reinfection as infected control mice. Histologic examination showed no visceral dissemination in either group. Failure of cortisone-treated mice to develop disseminated disease after reinfection did not appear related to the cortisone. Noninfected mice treated with cortisone were infected and a disseminated disease produced.

Discussion

The pathogenesis of chronic murine cytomegalovirus infection was investigated by serial virus assays and histological studies of infected C_3H mice. After intraperitoneal injection, cytomegalovirus replicated in the submaxillary glands reaching maximum titers within 3 weeks. After this time, the amount of virus rapidly decreased and by 7 weeks, virus or inclusions could rarely be found. Histologically, chronic inflammatory cells infiltrated the interstitium, surrounded and compressed the acini in which there were infected cells, and eventually permeated the acini to lyse infected cells. Degeneration of infected cells correlated with the decline of demonstrable virus in the submaxillary glands. It seems, there-

Table 1. Result of Injecting Fatal Dose of MCMV Into Mice Chronically Infected with MCMV*

	Cortisone-treated infected mice	Control mice	
		Infected	Noninfected
No. Injected	21†	12	4
Deaths	0	0	4

* Mice were initially injected 42 days previously with a sublethal dose of MCMV.

† Cortisone injections were begun 8 days after infection.

fore, that chronic infection in the submaxillary glands depends on the persistence of intact inclusion-bearing cells.

One problem of MCMV infection is the fact that infected cells persist considerably longer in the submaxillary glands than in the liver or spleen.[6] Infected cells in the submaxillary glands eventually degenerate, but, compared with infected cells in the liver and spleen of ICR[6] and C_3H mice,[9] it is delayed and associated with acinarrhexis. Histologically, the reason for the delayed degeneration seemed to be related to the reticulum network around the acini. Results of silver impregnation studies showed that acinarrhexis did not occur and infected cells did not degenerate, even in areas of heavy inflammation, until the argyrophilic reticulum network collapsed and fragmented.

Histologically, the correlation between intact infected cells and the integrity of the reticulum network suggests that this network acts as a barrier that temporarily prevents inflammatory cells from gaining access to infected cells in the acini. After inflammatory cells permeated the acini, infected cells rapidly degenerated just as they do in the liver and spleen.[6] Though the evidence is incomplete, this can explain chronic CMV infection in other organs, such as lacrimal glands and kidneys,[3] which also have a reticulum network.

Histologic studies also suggested that chronic inflammatory cells terminate chronic MCMV infection in the submaxillary glands by destroying infected cells. This is based on the observations that infected cells (1) only degenerated in the presence of interstitial inflammation, (2) did not degenerate or show evidence of pyknosis if the inflammatory reaction was inhibited with cortisone, and (3) after cortisone withdrawal, began to degenerate after inflammatory cells infiltrated the submaxillary glands. Degeneration of infected cells is probably related to the cytotoxic action of lymphocytes sensitized against virus-infected cells. We do not know if inflammatory cells exert a virucidal effect on extracellular CMV; this may be left for antibody.

Brodsky and Rowe [10] reported that random-bred Swiss mice shed CMV for life when infected as weanlings and Reubner *et al* [11] reported that chronic MCMV infection in the salivary glands can last for a year in Swiss Webster mice. In contrast, infectious virus and inclusions seldom persist after 7 weeks in the submaxillary glands of C_3H/Anf mice. Virus persists in the submaxillary glands much longer in ICR/HA mice than in C_3H mice. Histologically, inclusions persist longer in ICR mice and infected cells do not degenerate as rapidly in the presence of inflammation. The reasons why MCMV infected cells persist longer in the salivary glands of some strains of mice than others is unknown.

Despite persistent high virus titers in the submaxillary glands of cortisone-treated mice, there was no histologic evidence of visceral dissemination. Also cortisone-treated infected mice were resistant to reinfection with LD_{100} doses of MCMV while noninfected cortisone-treated mice could be infected with MCMV. This suggests that some protective mechanism, probably antibody, was present in the cortisone-treated mice which protected against reinfection and dissemination.

The studies reported here have not shed much light on the problem of viral activation during immunosuppression. Perhaps studies in other species of animals and with different strains of cytomegalovirus is indicated.

Summary

The murine cytomegalovirus (MCMV) causes a chronic infection in the submaxillary glands of C_3H/Anf and ICR/HA mice. After IP infection, virus titers reach peak levels in the submaxillary glands within 3 weeks. After this, virus titers decrease more rapidly in C_3H than in ICR mice. Decreasing titers are associated with interstitial inflammation in the submaxillary glands, acinarrhexis, and degeneration of infected cells. Inclusions and infectious virus persist longer in ICR mice. Special stains showed that as long as the reticulum network around the acini remained intact, the acini remained intact and infected cells did not degenerate. Penetration of acini by inflammatory cells was accompanied by fragmentation of the reticulum network, acinarrhexis, and degeneration of infected cells.

Administration of cortisone during chronic infection abolished the inflammatory reaction in the submaxillary glands. Virus titers did not decrease, but remained elevated at peak levels. Despite chronic cortisone administration, disseminated infection did not occur. Cortisone-treated, chronically infected mice were resistant to reinfection with lethal doses of MCMV.

The conclusions are made, chiefly from histologic observations, that chronic MCMV infection in the submaxillary glands depends on persistence of intact inclusion-bearing cells and that chronic inflammatory cells eventually terminate chronic infection.

It is proposed that the argyrophilic reticulum network in the submaxillary glands protects infected cells within the acini from interstitial inflammatory cells and that this is one mechanism of chronic cytomegalovirus infection in glandular organs.

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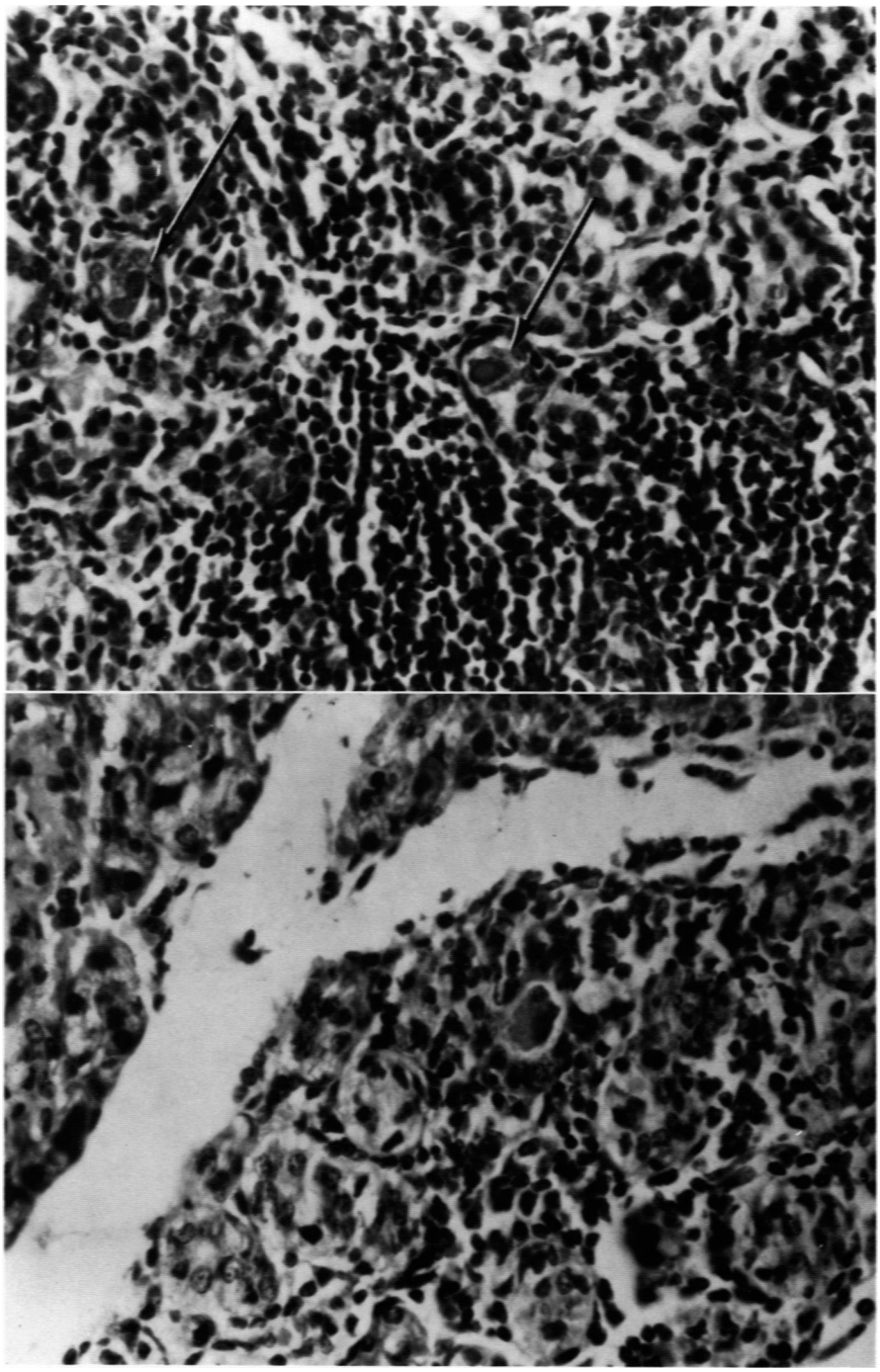
[Illustrations follow]

Legends for Figures

Fig 1. Infiltration of submaxillary glands by chronic inflammatory cells. Architecture is distorted by heavy inflammatory reaction. Many acini are compressed and obscured by dark staining inflammatory cells in lower half of field. *Arrows* indicate intranuclear inclusions. Day 25 after infection. H & E. × 332.

Fig 2. Focal inflammation and early degeneration of infected cell. Cell (center) stains deeply basophilic and appears to be undergoing pyknosis. Day 35 after infection. H & E. × 332.



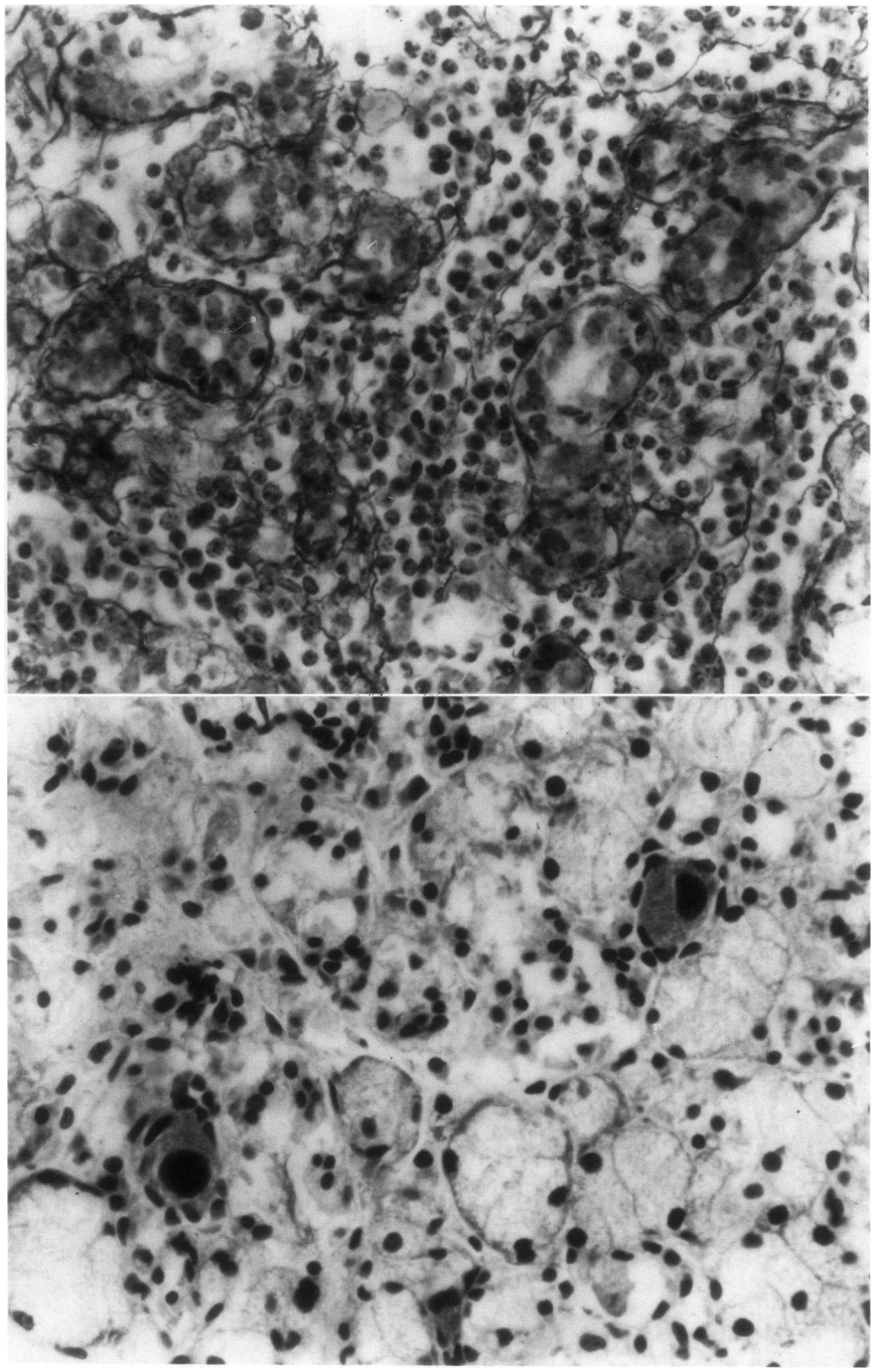


Fig 3. Reticulum stain of a control infected mouse with infiltration of inflammatory cells in submaxillary glands. Reticulum network is fragmented, disrupted, and no longer surrounds all acini. Many argyrophilic fibers are scattered among inflammatory cells. This photomicrograph should be compared with Fig 5. Day 35 after infection. Gridley. × 332.

Fig 4. Two prominent infected cells in submaxillary glands of cortisone-treated mouse. Note absence of inflammatory cells and preservation of glandular architecture. Day 49 after infection. H & E. × 400.

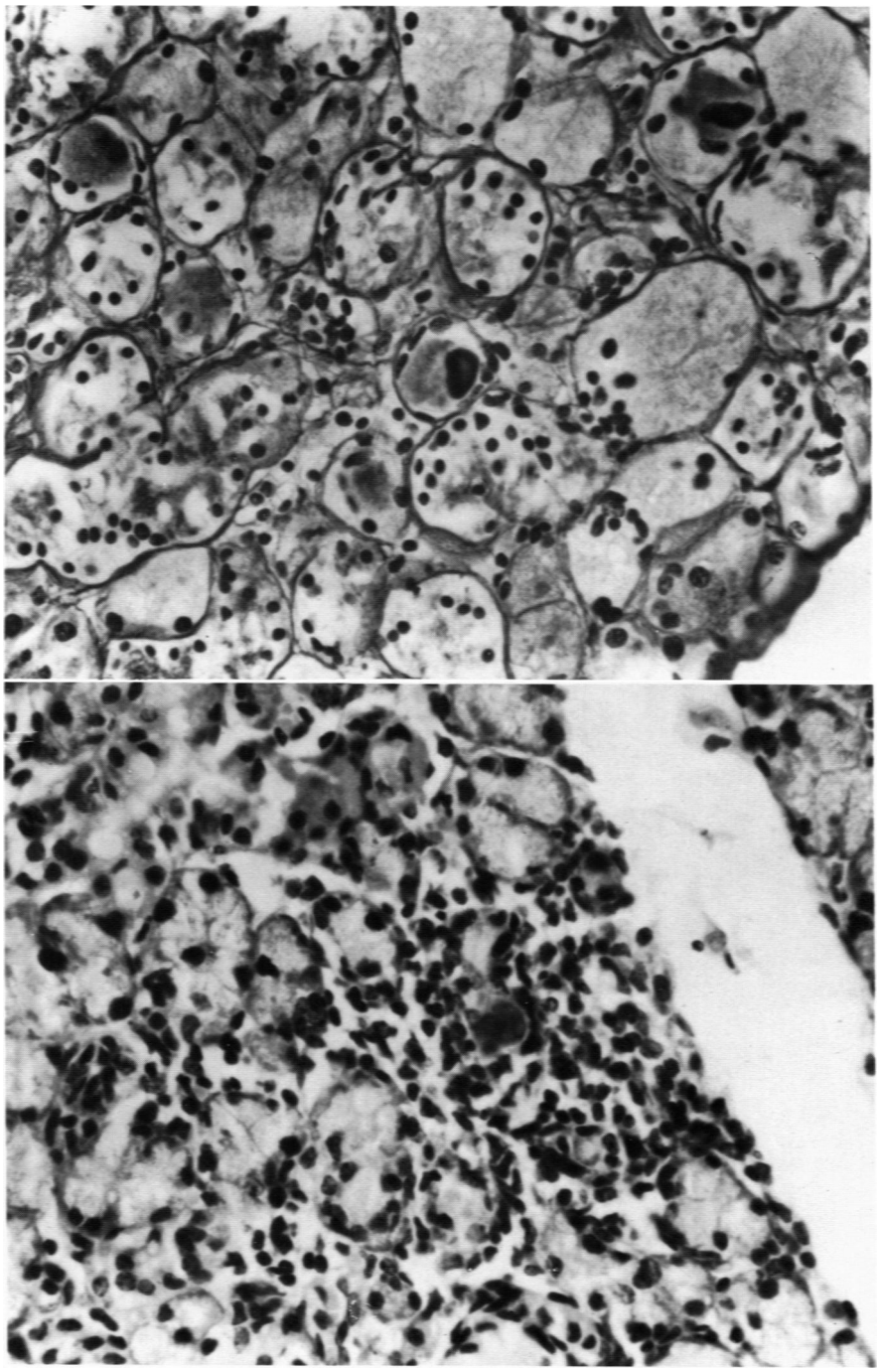


Fig 5. Reticulum stain of cortisone-treated mouse showing argyrophilic network around acini. Several infected cells are visible. Reticulum around acini with infected cells is intact. Day 49 after infection. Gridley. × 400.

Fig 6. Early inflammatory cell reaction around infected cell (center). Infected cell stains very basophilic and is undergoing degeneration. Cortisone-treated mouse, 21 days after discontinuing cortisone injections. H & E. × 375.