Experimental Yellow Fever Virus Infection in the Golden Hamster (Mesocricetus auratus). II. Pathology

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Subadult and adult hamsters were inoculated intraperitoneally with 10^6 TCID_{50} of yellow fever (YF) virus (Jimenez strain). Four animals from each group were subjected daily to histologic examination for 9 days. The liver showed spotty necrosis on day 3 after infection, which was followed by steatosis and focally confluent necrosis. In surviving hamsters, hepatocyte regeneration began on day 8, which was accompanied by decreasing steatosis. The spleen initially exhibited lymphoid hyperplasia, which was followed by lymphoid depletion and increased phagocytosis by splenic macrophages. Focal pancreatic acinar necrosis and spotty adrenal cortical necrosis were seen transiently between days 5 and 7. Viral antigen was detected immunohistochemically in the liver and the spleen. TUNEL analysis showed a dynamic change of hepatocyte necrapoptosis, with activity corresponding to the severity of disease. The histopathologic changes were more severe in younger (subadult) animals. The YF-hamster model appears to be an accurate and inexpensive experimental system for studying the pathophysiology and treatment of YF.

Despite the availability of a highly effective vaccine, yellow fever (YF) remains an important cause of morbidity and mortality in tropical regions of rural Africa and South America [1, 2]. Clinically, YF in humans can vary from a nonspecific illness to a fatal hemorrhagic fever. In mild cases, patients usually recover after an acute illness; in more severe or ultimately fatal cases, patients develop jaundice, increased serum transaminase levels, renal dysfunction, and hemorrhagic diatheses [3]. The YF case-fatality rate in Africa is estimated to be ~23% [3].

Our knowledge of the pathology of YF in humans comes mainly from descriptions of autopsies performed on patients who died of the disease; consequently, our knowledge is limited to changes that occur in the terminal stage (period of intoxication) of the disease [4–8]. More systematic pathologic descriptions come from studies of experimental infections in rhesus monkeys (Macaca mulatta), which is the animal model most commonly used for studies of the disease [9–20]. The clinical and laboratory findings in infected macaques are similar to those observed in severe human cases of YF, except that the course of the disease is more fulminant and usually fatal.

As reported in the accompanying paper [21], we have recently established a hamster model of YF that appears to be more representative of the spectrum of clinical and pathologic outcomes of YF virus infection in humans. The clinical, virologic, and immunologic findings in the YF-hamster model already have been described in the accompanying paper [21]. In this report, we describe the pathologic findings in hamsters examined at daily intervals after infection.

Materials and Methods

Animals and method of infection. The animals and method of infection were described in detail in the preceding, accompanying paper [21]. In brief, 2 age groups of female golden hamsters (Mesocricetus auratus) were used in the experiments reported in this paper. Thirty subadult (4–6 weeks old) and 36 adult (>6 weeks old) hamsters were inoculated intraperitoneally with 10^6 TCID_{50} of a hamster-adapted (designated as “p.10”) strain of the Jimenez YF virus. Four uninoculated hamsters of the same ages served as controls. In each of the experiments, 3 or 4 animals were killed daily for 8 or 9 days after inoculation. Blood samples were obtained before death for the measurement of viremia, antibody response, and liver-function tests; the results were reported in the preceding paper [21]. We performed necropsies on the animals immediately after death, and tissue samples were removed for subsequent histologic examination.

Antibodies for detection of YF viral antigen in hamster tissues. Mouse hyperimmune ascitic fluid (MIAF) prepared against the Asibi strain of YF virus was used to detect YF viral antigen in formalin-fixed, paraffin-embedded hamster tissue sections. The MIAF served as the primary antibody and was used at a dilution of 1:100 and was incubated overnight at 4°C, to obtain optimal staining.

Histologic preparation. Organs that were examined included the...
brain, the heart, lungs, the liver, the spleen, the pancreas, adrenal glands, kidneys, and the small and large intestines. Upon removal, the organs were fixed in 10% neutral buffered formalin for 24 h and then were transferred to 70% alcohol for storage. Samples subsequently were processed in an automatic tissue processor (Tissue-Tek VIP E300; Sakura) and were embedded in paraffin, after which, multiple histologic sections were made. All tissue samples were stained with hematoxylin-eosin, and selected special stains (Mason’s trichrome, reticulin, Oil Red O, and PAS with diastase) were used on some liver sections. For Oil Red O staining, liver samples were fixed in 10% buffered formalin, and then a sucrose technique was used for making cryostat sections. Sections for immunohistochemical staining were prepared on positively charged slides.

**Histopathologic evaluation.** The extent and severity of the pathologic changes in each organ were evaluated, using an Olympus BX40 compound microscope equipped with Olympus UPlanApo objectives. On the basis of preliminary observations, the following parameters were selected for semiquantitative evaluation of the histopathologic changes in liver and spleen. In liver sections, the degree of inflammatory infiltration, hepatic necrosis or apoptosis, and steatosis were evaluated by using the following scales: for inflammatory infiltration; 0, none; 1, occasional foci of inflammatory cell clustering; 2, more frequent foci but <10 foci per high-power field (HPF); 3, ≥10 foci per HPF; and 4, diffuse infiltration; for necrosis: 0, none; 1, occasional single necrotic cell; 2, <10 foci of single cell necrosis per HPF; 3, ≥10 foci of single cell necrosis per HPF; and 4, focally confluent necrosis; and for steatosis, a percentage of areas involved was evaluated. In evaluating changes in the spleen, we used lymphoid hyperplasia, lymphoid depletion, and splenic macrophage activation as the parameters; each was scored from 0 to 4 (0, normal; 1, minimal; 2, mild; 3, moderate; and 4, severe). Fewer specimens of pancreas, adrenal gland, and lungs were examined, so only a descriptive term was applied to these tissues.

**Immunohistochemical detection of YF viral antigen.** After deparaffinization, the formalin-fixed, paraffin-embedded tissue sections (3–4 μm in thickness) were immersed in 3% H2O2 for 10 min, to block endogenous peroxidase activity. This was followed by an antigen-retrieval heating step, using a citrate buffer (10% target retrieval solution; DAKO), at 90°C for 30 min. The YF-specific MIAF, described above and diluted 1:100, was incubated with the sections overnight at 4°C. To eliminate cross-species nonspecific reactions, an ISO-IHC AEC kit (InnoGenex) was used, according to the manufacturer’s instructions. To ensure uniform immunostaining conditions, tissue sections (liver, spleen, kidney, and, sometimes, adrenal and pancreas) from the same hamster were placed together on a single slide.

**In situ TUNEL assay.** An in situ apoptosis detection kit (ApopTag peroxidase kit; Intergen) was used, according to the manufacturer’s instructions, to stain sections of hamster tissue. In brief, after deparaffinization, tissue sections were treated with proteinase K, and then endogenous peroxidase activity was blocked by immersing the slides in 3% H2O2, for 5 min. This was followed by incubation with working-strength terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37°C for 1 h. The sections then were reacted with anti-digoxigenin–peroxidase conjugate for 30 min; 3,3-diaminobenzidine was used as the final color reaction product. Slides were counterstained with hematoxylin-eosin and were mounted with a cover slip for microscopic examination. For quantification, 10 random HPFs were examined, using a 40× objective, and apoptosis-positive nuclei were counted. Because of occasional nonspecificity of the in situ TUNEL assay, morphologically recognizable necrotic cells or foci were excluded from counting.

**Statistical analysis.** For the outcome measures of semiquantitative histologic parameters of liver and spleen, individual 2-way analysis of variance was used with days of infection (days 1–8) and age (subadult and adult) as factors to assess difference between centroids for significance. For each instance, the differences further were reflected in significant effects across both days of infection and age. An a priori omnibus, threshold (α rate of 0.05), was employed to correct for multiple factor comparison.

**Results**

**Histopathology**

**Liver.** On day 1 after infection, the liver appeared to be essentially normal in sections stained with hematoxylin-eosin. By day 2, occasional foci of mononuclear cell clustering had started to appear in some animals and were associated with individual degenerating hepatocytes (table 1). Acidophil bodies, some containing pyknotic nuclei resembling apoptosis, also were present. The frequency of these changes was increased on day 3 (figure 1A); by day 4, these changes were observed in all animals. Half the animals also showed focal inflammation of the portal tracts on day 3 after infection. The inflammatory infiltration remained essentially unchanged, but the spotty hepatocyte necrosis increased markedly in intensity on day 4.

On day 5, poorly developed microvesicular steatosis started to appear in many of the animals. It involved ~30%–40% of the lobular areas and was characterized by minute cytoplasmic vacuoles with indistinct borders (figure 1B). At this time, the spotty hepatocyte necrosis had increased to grade 3 or 4. Well-...
Figure 1. Photomicrographs of pathologic changes in liver from yellow fever (YF)-infected hamsters. A, Day 3 after infection, showing a focus of spotty necrosis (center of field) surrounded by mononuclear inflammatory cells. B, Day 5 after infection, showing more-severe inflammatory cell infiltration of the lobules, focal early steatosis, and hepatocytic necropoptosis. C, Day 6 after infection, showing diffuse, microvesicular steatosis of hepatocytes. Insert shows several hepatocytes with intranuclear viral inclusion bodies. D, Day 8 after infection, showing markedly decreased steatosis but diffuse increase in thickness of the hepatocytic cords or plates and brisk mitosis (arrowheads). E, Immunohistochemical staining for YF viral antigen on liver specimen from day 3 of infection, showing scattered positive (orange) cells. F, Immunohistochemical staining for YF viral antigen on liver specimen from day 6 after infection, showing much more diffuse distribution. G, In situ TUNEL staining of a liver specimen from day 4 after infection. Apoptotic cells are marked by positively stained (brown) nuclei. A–C and E–G, Original magnification, ×100; D, original magnification, ×50.
developed microvesicular steatosis was observed on day 6 and involved ∼85%–100% of the lobular areas (figure 1C). Inflammatory cell infiltration was more prominent on days 6 and 7 and appeared in both clustering and diffuse patterns, randomly involving the lobular parenchyma. The hepatocyte necrosis at this time was slightly less severe than that seen on day 5. Intracytoplasmic inclusion bodies resembling YF viral inclusions were evident in many hepatocytes (figure 1C, insert).

Lobular disarray, a feature of acute hepatitis, was seen in some animals on days 7 and 8 after infection (figure 1D). However, from day 8 onward, the number of animals exhibiting steatosis decreased; during this period, ∼20%–40% of the lobular areas were involved by a very mild or ill-formed microvesicular steatosis, mostly in zone 3. At the same time, there was increasing regenerative activity, manifested by brisk mitosis and marked thickening of hepatocytic plates (figure 1D), with decreased inflammation and reduced hepatocytic necrosis. By day 9 after infection, most of the hepatocytes looked normal, with a slight vesicular appearance and multiple prominent nuclei. Viral inclusion bodies rarely were seen. Livers of the infected hamsters generally were devoid of steatosis by day 9. In general, the degree of liver pathology was more severe in the younger (subadult) animals (table 1). A similar pattern was observed in the mortality rates of the subadult and adult hamsters after YF virus infection [21].

Spleen. Histopathologic changes in the spleens of infected hamsters consisted of lymphoid hyperplasia, initially characterized by expansion of the white pulp and followed by marked depletion. The depletion of lymphoid tissues was accompanied by lymphocytic necrosis and an increased number of foamy splenic macrophages, including tangible body macrophages (figure 2). The daily semiquantitative evaluation of these changes is summarized in figure 3.

Scattered lymphoid hyperplasia was seen on day 1 after infection but became more evident by the days 2 and 3. This change was characterized by expansion of the white pulp, with loss of the marginal zone lymphocytes (figure 2A). By day 4 or 5, there was mild lymphoid depletion and an increased number of splenic macrophages in the white pulp (figure 2B). The lymphoid depletion gradually increased to the maximum on days 6 or 7 (figure 2C), when only small islands of mononuclear cells were preserved in the spleen. Instead of small lymphocytes, plasma cells became more numerous in these areas. During this period of the infection, tangible body and foamy macrophages were present in both the white and red pulps; their distribution showed a gradual expansion from white to red pulp. In addition, shrunken cells with strongly eosinophilic, condensed cytoplasm and dark condensed nuclei similar to apoptotic bodies also were noted frequently. In general, the adult hamsters had more severe lymphoid hyperplasia than did the subadult animals, but the opposite was the case for lymphoid depletion and splenic macrophages (figure 3).

Pancreas. No pathologic changes were seen in the pancreas during the first 4 days of infection. However, on day 5, there was spotty parenchymal necrosis, which became more severe on days 6 and 7. It was characterized by numerous single necrotic cells in the acini (figure 2E). The islets of Langerhans were not involved. No pancreatic necrosis was observed on days 8 or 9.

Kidneys. Changes in the kidneys were inconsistent and were seen only in some animals. These consisted of degeneration of tubular epithelium, with diffuse swelling and collapse of the tubular lumens. The affected tubular epithelial cells had condensed, eosinophilic cytoplasm and dark condensed nuclei, which are features of early acute tubular necrosis (ATN). These changes were most evident in the medulla on days 1 and 2 but appeared to be transient. Full-blown ATN was never apparent, although occasional protein casts were seen in the tubular lumen. Mild swelling of tubular epithelium with collapse of the lumen was again noted in kidneys of some animals on days 6 and 7 after infection.

Adrenal gland. Between days 5 and 8 after infection, many hamsters showed mild-to-moderate necrosis of scattered single cells in the adrenal cortex (figure 2F). These changes gradually diminished with time (convalescence).

Lungs. No significant or consistent pathologic changes were identified in the lungs, except in 1 animal, which died on day 7 and showed diffuse hemorrhage and interstitial necrosis of the lung parenchyma.

Heart. In general, no specific or consistent pathologic abnormalities were seen in the endocardium, myocardium, or epicardium. In 2 animals, mild, nonspecific myocardial degeneration was observed, but the significance of this finding was not clear.

Other organs. A single ovary was included with tissues submitted from a hamster on day 5 after infection. Microscopic examination of this organ revealed prominent, spotty necrosis in the parenchyma.

Detection of YF Viral Antigen

Sections of liver, spleen, and kidney (3–4-μm thick) were prepared by immunohistochemical techniques, as described in Materials and Methods. YF-specific antigen was detected readily in tissue sections of both the liver (figure 1E, 1F) and the spleen (figure 2D), but was not identified in the kidneys (not shown).

Status of Parenchymal Cell Apoptosis in the Liver, as Detected by TUNEL Assay

Liver specimens of 3 hamsters from each day after infection were stained with the in situ apoptosis detection kit, and cells with positive nuclear staining (figure 1G) were counted in 10 random HPF. The results were used to determine an apoptosis index, which was expressed as the average number of positive cells per HPF (40× objective). The mean apoptosis index in the livers of 3 hamsters from each day after infection are shown in figure 4.
Figure 2. Photomicrographs of pathologic changes in spleen, pancreas, and adrenal gland from yellow fever (YF)-infected hamsters. A. Spleen from day 3 after infection, showing enlargement of lymphoid follicles with diminishing marginal zone. B. Spleen from day 5 after infection, showing shrinking of the lymphoid follicles (white pulp), with increased number of splenic macrophages. C. Spleen from day 6 after infection, showing nearly complete disappearance of the lymphoid follicles in some areas, replaced by macrophages and small clusters of plasma cells. D. Immunohistochemical staining for YF viral antigen on a spleen specimen from day 5 after infection, showing strong cytoplasmic staining (orange). E. Spotty necrosis of the acinar parenchyma in pancreas of a hamster on day 6 after infection. F. Spotty necrosis in the adrenal cortex of a hamster on day 8 after infection. A–C. Original magnification, ×50; D–F, original magnification ×100.
Figure 3.  Semiquantitative analyses of histopathologic changes in spleens of yellow fever–infected hamsters. Changes were scored from 0 to 4: 0, normal; 1, minimal; 2, mild; 3, moderate; and 4, severe. Difference of each parameter between the subadult and adult groups is statistically significant (P < .05, 2-way analysis of variance). Open bars, subadult hamsters; solid bars, adult hamsters.

Discussion

In patients dying of YF, abnormalities have been described in multiple organs; however, the most consistent findings are in the liver [4, 6–8, 19, 22]. Grossly, the liver is usually enlarged and has a mottled yellow (“boxwood”) appearance. It is often friable and fatty [19]. Microscopically, the most striking finding is a midzonal, or diffuse, coagulative necrosis of the hepatocytes. Depending on the severity and/or length of illness, the necrosis may be manifested as scattered individual acidophilic bodies (Councilman bodies) or as larger areas of confluent ac-

idophilic changes in the hepatocytes. A “salt-and-pepper” pattern within each lobule also has been described during earlier stages of the disease [8], which probably is due to the apoptosis of hepatocytes, with nuclear debris in the cytoplasm of the dying cells. During the earlier stages of the disease and before massive acidophilic changes occur, microvesicular steatosis also may be seen [19]. Inflammatory cell infiltration is usually minimal or absent in fatal human cases and is not directly associated with the necrosis. There is generally no collapse of the reticulin framework or postnecrotic fibrosis [2], which is consistent with an acute process. These pathologic features are quite specific for YF and have been described as “pathognomonic” when encountered under conditions compatible with the disease [8].

The kidneys in fatal YF cases are often enlarged, congested, and icteric [3–5, 7, 8]. Microscopic findings include cloudy swelling, fatty change, and necrosis of tubular epithelium, with granular and hyaline intratubular casts [19]. The spleen is usually enlarged, congested, and firm, often with prominent Malpighian bodies. Microscopically, it typically exhibits congestion with “small lymph nodules” and a prominence of “endothelial leukocytes” [19]; the latter most likely represent macrophages. Cardiac involvement is grossly manifested by right ventricular dilatation, subpericardial hemorrhages, and icterus. Histologic changes in the heart are usually subtle and include cloudy swelling, degeneration, fatty infiltration, and necrosis of myofibers [23, 24]. Changes in the brain are relatively mild and nonspecific, consisting of perivascular hemorrhage and edema. The lungs may exhibit recent hemorrhagic changes and congestion but usually show little inflammatory reaction [19].

In rhesus monkeys experimentally infected with YF virus, the principal finding during the first 3 days is an “eosinophilic necrosis of Kupffer’s cells” in the liver [10, 11]. This pathologic process also has been observed in “reticuloendothelial cells” of the spleen, lymph nodes, and glial cells of the brain. In the liver,
mixed macro- and microvesicular steatosis also are evident before and during the period when parenchymal necrosis occurs [18]. In the rhesus-YF model, progressive degeneration and necrosis of hepatocytes become evident ~3 days after infection and reach a peak on day 5 [11, 16]. The hepatocytes of some animals also exhibit gradual glycogen depletion [16, 18]. Immunofluorescent studies indicate that the necrosis correlates with the accumulation of viral antigen in the hepatocytes [10]. In addition, intranuclear inclusions also may be present in the hepatocytes.

The spleens of infected monkeys show gradual depletion of lymphoid follicles [11], which correlates with a decrease in the number of peripheral lymphocytes [11, 14]. Monath et al. [14] reported acute necrosis of lymphoid cells in the germinal centers (which represent B cell regions) of spleens of rhesus monkeys infected with YF virus. This prominent germinal center lymphocytic necrosis also was seen in peripheral lymph nodes, as was sinus histiocytosis [14]. Changes in the kidneys of YF-infected monkeys usually were not evident until shortly before death (<24 h) and were manifested morphologically by granular degeneration and, in some cases, by tubular necrosis, mostly of the convoluted tubules [14]. Eosinophilic proteinaceous casts also were seen in the tubules. In the heart, granular degeneration and necrosis of myocardial fibers are the main findings, which Lloyd [23] suggested were mediated by direct viral injury.

The most striking histopathologic findings in hamsters infected with YF virus were in the liver, spleen, and pancreas. Collectively, these changes (especially in the liver and the spleen) were very similar to those in patients infected with YF who died and in experimentally infected macaques, as described elsewhere [4, 7, 8, 11, 14, 17–19, 22].

As observed in previous serial studies of YF-infected rhesus monkeys [10, 11, 14], massive hepatocyte necrosis did not occur in the YF-infected hamsters until day 4 after infection. The appearance of marked hepatocyte necrosis in the hamsters correlated well with the increase in serum levels of aspartate and alanine aminotransaminase, which also started to elevate on day 4 after infection [21]. On day 5, microvesicular steatosis (an indication of altered liver function and abnormal lipid metabolism) was observed. This histopathologic change likewise correlated with an increase in serum bilirubin levels and coagulation abnormalities in the blood of infected hamsters [21]. Death in the infected hamsters, as in humans and macaques, usually occurred between days 5 and 7 of illness. In surviving hamsters, hepatocyte regeneration was apparent by day 8, as the degree of steatosis decreased. These latter changes correlated with the biochemical indicators of improved liver function (decreasing serum transaminase and bilirubin levels), which occurred at the same time [21].

Inflammatory cell infiltration of the liver parenchyma was observed in histologic sections from infected hamsters on days 5–7. This correlated with maximum lymphoid depletion in the spleen, evidence of increased phagocytic activity, and elevation of the peripheral white blood cells [21]. During this same period, the serum levels of YF hemagglutination-inhibiting antibodies in the infected animals also increased rapidly [21].

The histopathologic changes in the spleens of YF-infected hamsters began as a reactive lymphoid hyperplasia but were quickly followed by necrosis of lymphocytes, which led to depletion of the white pulp. These changes resembled those in humans and monkeys, as described elsewhere [4, 7, 8, 14, 18]. In macaques, the depletion of lymphoid tissue has been described as “progressive diminution of lymphocytes, with concurrent increase in large mononuclear cells” and “reticuloendothelial cell hyperplasia” [4]. Morphologically, these descriptions represent the same processes that we observed in the hamster spleens. The “large mononuclear cells” described in earlier monkey studies probably correspond to the increased number of plasma or plasmacytoid cells seen in our hamster model, and the “reticuloendothelial cell hyperplasia” is probably the increase in splenic macrophages that was observed in the infected hamsters.

Histologic changes in the kidneys of infected hamsters were not consistent and may reflect artifactual changes rather than specific changes directly related to YF virus infection. Immunohistochemical stains did not demonstrate the presence of YF antigen in the kidneys. These negative results are credible because the kidney sections were placed on slides with sections of liver and spleen from the same animals, and many of the latter were positive for YF viral antigen.

In immunohistochemical studies, the intensity of staining for YF viral antigen was greater in the spleen than in the liver of infected hamsters. This finding is compatible with the marked lymphocytic necrosis observed in the spleen. Lymphocytic necrosis was noted to be a prominent feature of YF pathology in experimentally infected macaques, involving lymph nodes, the spleen, tonsils, and Peyer’s patches [14]. Similar findings have been described in the spleens of human YF patients [19]. Because of the focus on viscerotomy and liver histopathology as diagnostic tools in YF, other tissues often are not collected or examined in suspected human cases of the disease. Monath et al. [14] suggested that lymphoid tissue may be a major target of YF virus and that the diagnostic importance of this organ may be overlooked. Results of our hamster model support this view and indicate that further studies on the role of lymphoid tissue and the reticuloendothelial system in YF pathogenesis are warranted.

Histopathologic studies of livers of the YF-infected hamsters showed prominent acidophil bodies, with morphology ranging from acidophilic intracytoplasmic granules to conspicuous Councilman bodies free in the sinusoids. A similar histopathologic picture has been described in the livers of patients infected with YF who died and of experimentally infected macaques. The morphology of these acidophil bodies is compatible with apoptotic bodies, which suggests that cell death in the YF-infected liver may start with cell apoptosis that is triggered by the infecting virus. The results from our in situ TUNEL staining support this concept. However, it is unclear at this stage whether
some component of the virus directly triggers apoptosis or whether humoral antibodies or cellular immune factors induced by the virus infection secondarily trigger the process. Hepatocytic apoptosis has been demonstrated in vitro in a human hepatoma cell line (Hep G2) infected with YF virus: apoptotic hepatocytes started to appear within 48 h after infection, and the number increased with the length of infection [25]. In the in vitro Hep G2 model, no other cell populations were present, which suggests that YF virus may trigger the apoptosis directly rather than indirectly through a secondary effect on inflammatory cells. If the virus acts directly on hepatocytes, then the inflammatory cell infiltration observed on days 6 and 7 after infection in our hamster model may simply represent a host response to “clean-up” cells that undergo apoptosis or necrosis. Obviously, much is still unknown about the pathogenesis and pathophysiology of YF, but the availability of hamsters offers a new and relatively inexpensive animal model to address some of these questions.

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References