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Aged BALB/c Mice as a Model for Increased Severity of Severe Acute Respiratory Syndrome in Elderly Humans

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Advanced age has repeatedly been identified as an independent correlate of adverse outcome and a predictor of mortality in cases of severe acute respiratory syndrome (SARS). SARS-associated mortality may exceed 50% for persons aged 60 years or older. Heightened susceptibility of the elderly to severe SARS and the ability of SARS coronavirus to replicate in mice led us to examine whether aged mice might be susceptible to disease. We report here that viral replication in aged mice was associated with clinical illness and pneumonia, demonstrating an age-related susceptibility to SARS disease in animals that parallels the human experience.

Since the severe acute respiratory syndrome (SARS) outbreak of 2002 and 2003 was recognized, there has been a need to identify appropriate animal models in which pathogenesis and preventive strategies can be evaluated. Several animal species support replication of SARS coronavirus (SARS-CoV) (14, 16, 17, 27, 29, 33) and are useful as models for the evaluation of treatment and prophylaxis against SARS-CoV (1, 4, 5, 12, 30, 34). However, viral infection found in animal models is accompanied by different degrees of pathology, and reports of associated clinical illness are inconsistent. Although SARS-CoV replicates in the respiratory tracts of 4- to 6-week-old BALB/c mice without associated signs of clinical illness or overt pathology (29), the heightened susceptibility of elderly humans to severe SARS (2, 6, 8, 15, 22, 24, 32) led us to hypothesize that aged mice might be more susceptible to the disease than young mice. We report here a mouse model that demonstrates consistent signs of clinical illness, supports high levels of SARS-CoV replication, and displays histopathological lesions similar to those observed in human cases of SARS (9, 13, 20, 23, 28, 31).

We administered 10^5 50% tissue culture infective doses (TCID₅₀) of SARS-CoV (Urbani isolate [13]) intranasally to 12- to 14-month-old BALB/c mice as previously described (29). SARS-CoV-infected aged mice demonstrated signs of clinical illness characterized by significant weight loss, hunching, ruffled fur, and slight dehydration measured by skin turgor. Weight loss began 3 days postinfection (p.i.), with a nadir of 8% loss on day 4 p.i., and was noted through day 6 ($P < 0.04$)

(Fig. 1A). Clinical signs of illness resolved by day 7 p.i., and inactivity, changes in gait, and mortality were not observed.

The levels of viral replication in the lungs, nasal turbinates, liver, and spleen were analyzed from mice that were euthanized (four mice per day) on days 2, 5, 9, and 13 p.i. Supernatants of 10% (wt/vol) tissue homogenates were titrated on Vero cell monolayers as previously described (29). Virus was detected in lungs at high titers ($\sim 10^8$ TCID₅₀/g) as early as day 2 p.i., and titers remained high ($> 10^7$ TCID₅₀/g) on day 5 p.i. Virus was also recovered from the upper respiratory tract (nasal turbinates) and the liver at days 2 and 5 p.i. (Fig. 1B). Virus was not detected in whole blood (assayed at 48 and 72 h p.i.; the limit of detection was $10^{1.5}$ TCID₅₀/ml) or in spleen.

Following SARS-CoV or mock inoculation, mice were euthanized on days 1, 2, 3, 5, 9, and 13 p.i., and lungs and nasal turbinates were fixed with 10% formalin and processed for histopathological and immunohistochemical (IHC) examination as previously described (13, 17, 28, 29). Soon after infection (days 1 to 3 p.i.), SARS-CoV antigens were detected by IHC staining in ciliated, columnar epithelial cells of the nasal turbinates and bronchioles (Fig. 2A and B, respectively) and in alveolar pneumocytes (Fig. 2B). IHC staining showed SARS-CoV antigen associated with epithelial necrosis and abundant necrotic debris in airways (Fig. 2C to F). At day 3 p.i., loose collections of mixed perivascular infiltrates comprised predominantly of lymphocytes and histiocytes were noted around vessels adjacent to bronchioles (Fig. 2C). On day 5 p.i., infected pneumocytes were still detectable (Fig. 3A) but in fewer numbers than at day 3 p.i. In contrast, perivascular infiltrates, first noted on day 3 p.i., were more prominent at day 5 p.i. Viral antigens were not detected by IHC staining in respiratory tissues after day 5 p.i. Changes indicative of alveolar damage,

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FIG. 1. Clinical illness in aged BALB/c mice during SARS-CoV infection. (A) Mean percentages of change in weight for mock-infected (open squares) ($n = 5$) or SARS-CoV-infected (filled squares) ($n = 6$) mice. Error bars indicate standard errors. The results of one of four replicates (mean weight loss of 6 to 10%) are presented. Asterisks indicate statistically significant weight loss ($P < 0.045$). The mean initial weight of mice (at day 0) was 26.9 ± 0.5 g. Various findings for the lungs of mice infected with SARS-CoV are indicated below the graph. Plus signs indicate the presence of a finding, minus signs indicate the absence of a finding, and blanks indicate that no evaluation was made at that time point. (B) Replication of SARS-CoV in various tissues following intranasal inoculation. The mean virus titer is expressed as the $\log_{10}$ TCID₅₀ per gram of tissue ($n = 4$). Filled bars, lungs; gray bars, nasal turbinates; open bars, liver; hatched bars, spleen. The limit of detection was $10^{1.5}$ TCID₅₀/g of tissue (dashed line). Error bars indicate standard errors. All animal experiments were approved by the National Institutes of Health Animal Care and Use Committee, all work with infectious virus was performed inside a biosafety cabinet in a biosafety containment level 3 facility, and personnel wore powered air-purifying respirators (HEPA AirMate; 3M, Saint Paul, Minn.).

including multifocal, interstitial, and predominantly lymphohistiocytic infiltrates, proteinaceous deposits around alveolar walls, and intraalveolar edema, were seen beginning on day 5 p.i. (Fig. 3B). At day 9 p.i., perivascular infiltrates persisted, and the changes associated with alveolar damage were accompanied by a proliferation of fibroblasts in inflammatory foci (Fig. 3C). The number and size of these foci decreased over time, but a few persisted in the lungs of some mice for at least 29 days p.i. It is possible that these foci (Fig. 3D) in SARS-CoV-infected mice represent histopathologic correlates of fibrosis or scarring identified by high-resolution computed tomography scanning of the lungs of some human patients who have recovered from severe cases of SARS (7, 19).

Given the evidence of viral replication and inflammation in the lungs, we examined which inflammatory mediators were

present. Aged BALB/c mice were euthanized (four mice per day), and tissues were collected on days 1, 2, 3, 5, 9, and 13 p.i. Supernatants of 20% (wt/vol) lung homogenates were analyzed in duplicate by enzyme-linked immunosorbent assay for the following cytokines per manufacturer protocols: alpha interferon (IFN- α ; PBL Biomedical Laboratories, Piscataway, N.J.), IFN- γ , tumor necrosis factor alpha (TNF- α), interleukin 4 (IL-4), IL-10, and IL-12 (Quantikine Immunoassays; R&D Systems, Minneapolis, Minn.). Lungs from two mock-infected, age-matched mice were collected for determination of baseline cytokine levels in aged BALB/c mice. Levels of IFN- α , IFN- γ , and TNF- α were elevated (>2 -fold increase over levels in mock-infected control animals) in SARS-CoV-infected aged mice at 2 and 3 days p.i. during peak viral replication (Fig. 4A to C). Slight elevations in IFN- γ and TNF- α were also ob-

FIG. 2. Histopathological and immunohistochemical findings for aged BALB/c mice 2 to 3 days following SARS-CoV infection. (A) SARS-CoV antigens (arrowheads) in ciliated columnar epithelium of nasal turbinates (day 2 p.i.); (B) SARS-CoV antigens (arrowhead) in the cytoplasm of bronchiolar epithelium and alveolar pneumocytes (day 2 p.i.); (C) necrosis and sloughing of respiratory epithelial cells in a bronchiole and adjacent, predominantly lymphohistiocytic, inflammatory cell infiltrates (day 2 p.i.); (D-F) extensive cellular debris in airway lumen comprised of necrotic epithelium and inflammatory cells (arrow) with abundant staining of SARS-CoV antigens (arrowheads) (day 3 p.i.). Antigens were detected with hyperimmune mouse anti-SARS CoV ascitic fluid at a 1:1,000 dilution. Stains used were immunoalkaline phosphatase with naphthol-fast red substrate and hematoxylin counterstain (A, B, E, and F) and hematoxylin and eosin stain (C and D). Original magnifications, $\times 50$ (A-E) and $\times 100$ (F).

FIG. 3. Histopathological and immunohistochemical findings in aged BALB/c mice 5 to 13 days following SARS-CoV infection. (A) SARS-CoV antigens (arrowhead) in alveolar pneumocytes (day 5 p.i.); (B) focus of early alveolar damage showing intraalveolar edema (large arrows) and interstitial, predominantly lymphohistiocytic, inflammatory cell infiltrates (day 5 p.i.); (C) lymphohistiocytic inflammatory cell foci accompanied by fibroblast proliferation (small arrows) in pulmonary parenchyma (day 9 p.i.); (D) focus of residual alveolar damage in predominantly normal lung (day 13 p.i.). Antigens were detected with hyperimmune mouse anti-SARS-CoV ascitic fluid at a 1:1,000 dilution. Stains used were immunoalkaline phosphatase with naphthol-fast red substrate and hematoxylin counterstain (A) and hematoxylin and eosin stain (B-D). Original magnifications, $\times 100$ (A), $\times 25$ (B, C), and $\times 12.5$ (D).

served on day 9 p.i. after the peak of viral replication. However, the other cytokine levels were not elevated greater than twofold over mock levels at any time point assayed (Fig. 4D to F).

A number of defects in innate and adaptive immune responses have been described to occur during immune senescence in mice and humans (3, 10, 18, 21, 25, 26, 35). Glass et al. (11) studied the mechanisms underlying the clearance of SARS-CoV from the lungs of young C57BL/6 (B6) mice and inferred that NK cells and adaptive cellular immunity do not play a role in clearance because Beige, $CD1^{-/-}$, and $RAG1^{-/-}$ mice that selectively lack NK cells, NK-T cells, and T and B lymphocytes, respectively, were able to clear virus as rapidly and completely as normal young B6 (11) and BALB/c (29) mice. Aged BALB/c mice developed mean serum neutralizing SARS-specific antibody titers of 1:14 and 1:38 by 3 and 5 weeks p.i., respectively, which is well within the range of titers seen in

young SARS-CoV-infected mice, indicating that aged mice are as capable as young mice of mounting an adaptive immune response to SARS-CoV infection. However, in contrast to young BALB/c and B6 mice, for which elevations in proinflammatory cytokines, clinical illness, and histopathological changes following SARS-CoV infection were not observed (11, 29), SARS-infected, aged BALB/c mice showed elevated levels of IFN- α , IFN- γ , and TNF- α early in infection. This observation suggests that a proinflammatory cytokine response may be responsible for subsequent disease-associated events. Further exploration of the components of innate and cell-mediated immunity in aged mice, including the presence or absence of various chemokines, is warranted to elucidate the pathogenesis of SARS-associated disease and the mechanism for viral clearance.

In conclusion, we present here the first demonstration of

FIG. 4. Pulmonary cytokines detected by enzyme-linked immunosorbent assay in SARS-CoV-infected aged BALB/c mice. SARS-CoV-infected mice (shaded bars) (four mice per group) demonstrated elevated levels of cytokines IFN- α (A), IFN- γ (B), and TNF- α (C) compared to mock-infected, age-matched controls (open bars) (two mice per group) at days 2 and 3 p.i. Consistent rises in cytokine levels, defined as a >2 -fold increase over mock levels for more than a single time point, were not observed for IL-10 (D), IL-12 (E), and IL-4 (F). Y-axes indicate pg of measured cytokine per g lung tissue. X-axes indicate days after inoculation with SARS-CoV. Bars indicate geometric means within each group. Samples were assayed in duplicate, and open circles represent mean values of duplicates. Error bars indicate standard errors of geometric means.

age-related susceptibility to SARS-CoV disease in animals that parallels the human experience with SARS. Replication of SARS-CoV is enhanced and prolonged in 12- to 14-month-old BALB/c mice compared to that in young mice, and the enhanced viral replication is accompanied by evidence of clinical illness, alveolar damage, and interstitial pneumonitis. Elevation of proinflammatory cytokines is also observed in SARS-infected, but not in mock-infected, aged mice. The aged-mouse model will facilitate research into the pathogenesis of SARS and represents a critical addition to the models that are available for SARS prevention and treatment studies.

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