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Authors: Fox, Jessica, Bergeron, Marie-Eve, and Haston, Christina K.

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Genetic Deficiency in Complement Component 4b Does Not Alter Radiation-Induced Lung Disease in Mice

Jessica Fox, Marie-Eve Bergeron and Christina K. Haston

Department of Medicine and the Meakins-Christie Laboratories, McGill University, 3626 St. Urbain Montreal, Quebec, Canada

INTRODUCTION

Thoracic cavity radiotherapy can produce serious inflammatory (alveolitis) or fibrotic (fibrosing alveolitis) side effects in the lung (1, 2). Pulmonary fibrosis is characterized by cellular proliferation and progressive accumulation of extracellular constituents, which result in remodeling of the lung interstitium (3). Alveolitis, or pneumonitis, is an inflammatory response associated with cellular infiltration of the airspace and thickening of the alveolar walls (3).

The exact mechanisms, which lead to both alveolitis and fibrosis, are as yet unknown, but a component of the innate immune system, complement, may be involved in the lung response to thoracic irradiation. Clinical implication of complement in the pulmonary phenotype includes a proteomic analysis that reveals complement component C3 and C4b binding protein alpha chain and vitronectin of 76 proteins analyzed to be more highly expressed in the plasma of lung cancer patients with radiation-induced lung toxicity grade ≥ 2 compared to those without (4). Studies in animal models that exhibit radiation-induced alveolitis and fibrosis phenotypes with similar temporal and histological patterns to the clinical response (1, 2) also suggest a complement contribution to lung disease. Specifically, we (5–7) and others (8, 9) have reported that C57BL/6J mice respond to high-dose whole-thorax irradiation by developing alveolitis and atelectatic regions of fibrosis at approximately 6 months after radiation treatment. We also defined the pulmonary response to 18 Gy whole-thorax irradiation, at the expression level in mice of three strains, A/J, C3H/HeJ (alveolitis response) and C57BL/6J [fibrosis response, ref. (10)] and identified the pathway of complement signaling to be significantly represented only in the fibrosis responding strain.

In this study, we exposed C4b knockout mice (11) to whole-thorax irradiation to determine whether a deficiency in this complement component altered the development of radiation-induced lung disease.

MATERIALS AND METHODS

Mice

C57BL/6-C4b knockout mice (strain name B6.129S4-C4b<sup>−/−</sup> /J; genetic background C57BL/6J, Jackson Laboratory stock no. 003643) and wild-type littermates were obtained from the Jackson Laboratory and were bred and housed in the animal facility of the Meakins-Christie Laboratories. The mice were genotyped for genetic background C57BL/6J (alveolitis response) and C57BL/6J [fibrosis response, ref. (10)] and identified the pathway of complement signaling to be significantly represented only in the fibrosis responding strain.

Female mice were treated at 8 weeks of age. Lung damage was elicited by whole-thorax radiation exposure using a GammaCell
Cesium-137 unit, as previously described (5, 7). A dose of 18 Gy delivered at 0.6 Gy/min, was given to elicit a lung disease response in the majority of the animals based on the known response of the background strain (5–7, 10). The rest of the body was shielded with 3 cm of lead to reduce the beam strength to 3% in this area. The irradiated mice were humanly euthanized when moribund. For this assessment the mice were weighed weekly beginning nine weeks after irradiation; animals that lost >20% of their body weight within a period of 2 weeks and exhibited distress through ruffled fur, accelerated breathing and hunched posture were euthanized, as described previously (5). The survival time of a mouse was either the time at euthanization due to moribund symptoms, which were evident in the majority of mice, or the time at which the animal was found dead. Differences in survival between strains of mice grouped by C4b genotype were assessed by the log-rank test. The control mice were not treated and were sacrificed at the 15–24 week time points.

**RESULTS**

**Survival Time and Weight Change Phenotype**

To determine whether mice deficient in C4b differ from wild-type littermates in their response to whole-lung irradiation, mice of each genotype were exposed to a dose of radiation of 18 Gy and sacrificed upon presentation of respiratory distress. As shown in Fig. 1, postirradiation survival time of C4b–/– mice did not differ from that of wild-type littermate mice (P = 0.28). Further, the onset of distress was similar in the groups of mice, as indicated by postirradiation weight loss as shown in Fig. 2.

**Histological and Biochemical Phenotype**

Histological evaluation of the lungs, which was completed at the times indicated in Fig. 1, revealed that the respiratory distress of the irradiated mice was due to

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**FIG. 1.** Postirradiation survival time of wild-type and C4b-deficient mice. Mice (n = 11–15 per group) were exposed to a single dose of 18 Gy whole-thorax radiation and euthanized when in respiratory distress.

**FIG. 2.** Postirradiation body weight of wild-type and C4b-deficient mice. Mice were exposed to a single dose of 18 Gy whole-thorax radiation and body weight was recorded beginning at week 9 after irradiation, mean (± standard error). There were no significant differences in body weight in irradiated mice grouped by C4b genotype, P > 0.16.

**Hydroxyproline Assay**

The hydroxyproline content of the right mouse lungs was determined using previously described standard methods (13–15).

**Bronchoalveolar Lavage Fluid (BAL) Analysis**

The BAL fluid was centrifuged (302g for 10 min at 4°C) and the supernatant was removed and stored at −85°C. The cellular pellet was resuspended in 0.25 mL PBS. Inflammatory cell counts were performed (400× magnification) on cytocentrifuged cells (214.2g for 3 min) after staining with a hematoxylin-eosin kit (Hema-3 Stain Set by Protocol), as previously described (5, 7).
fibrosing alveolitis (Fig. 3) and is in agreement with previous reports of the wild-type strain (5–10). Image analysis of histological sections demonstrated that C4b−/− mice also developed significant fibrosis after thoracic irradiation, but was similar to the level observed in wild-type mice (Fig. 3B). As a second indicator of fibrosis, we measured the hydroxyproline levels in the lungs of untreated and irradiated mice. The tissue level of hydroxy-
Inflammatory Cell Counts

To determine whether the inflammatory response to whole-thorax irradiation is altered by a C4b deficiency, we quantified the bronchoalveolar cell types. There were radiation-induced increases in total bronchoalveolar lavage cell counts (data not shown) in agreement with previous reports of the wild-type strain (5, 7), but as shown in Fig. 4, there was no difference in lavage cell differential by C4b genotype in radiation-treated mice. The lavage differential of unirradiated mice did not differ by C4b genotype (P > 0.25, data not shown).

**FIG. 4.** Bronchoalveolar lavage cell differential of wild-type and C4b-deficient mice following whole-thorax radiotherapy. Mice were treated with 18 Gy to the lung and sacrificed upon presentation of respiratory distress. Bronchoalveolar lavage samples were collected at necropsy and cells were morphologically identified from cytospin preparations. The average percentage of each cell type (±std) is presented. There were no significant differences in lavage cell differential in irradiated mice grouped by C4b genotype, P > 0.21.

In summary, we evaluated the effect of C4b deficiency on radiation-induced lung disease in a mouse model and show that the deficiency does not significantly alter survival time nor the extent of histological disease evident in distressed mice. These results therefore indicate that radiation-induced lung disease is not affected by a C4b deficiency in C57BL/6J mice.


