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Recombinant Human Hepatitis B Vaccine Initiating *Alopecia Areata*: Testing the Hypothesis Using the C3H/HeJ Mouse Model

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Abstract

Untoward effects of human vaccines suggest that recombinant hepatitis B vaccine may induce *alopecia areata* (AA) in some patients. Similar untoward immunological effects may also account for AA-like diseases in domestic species. In this study the C3H/HeJ spontaneous adult onset AA mouse model was used to test the role, if any, of recombinant hepatitis B vaccine on the initiation or activation of AA. Initial experiments demonstrated no effect on induction of AA in young adult female C3H/HeJ mice ($p = 0.5689$). By contrast, older females, those at the age when AA first begins to appear in this strain, had a significant increase ($p = 0.0264$) in the time of onset of AA suggesting that the vaccine may initiate disease in mice predisposed to AA. However, larger vaccine trials, which included diphtheria and tetanus toxoids as additional controls, did not support these initial result findings and suggest that AA associated with vaccination may be within the normal background levels of the given population.

Introduction

Hair loss (alopecia) is only rarely reported following receipt of vaccines in many species. Fewer than five cases of alopecia were reported through the Vaccine Adverse Events Reporting System in humans as of 1996. Even when hair loss is reported after vaccination, it usually is mild and self-limiting. However, because hair loss can occur through autoimmune mediated mechanisms, it may be a marker for other immunologically mediated vaccine effects. An analysis of a series of sixty human cases of hair loss occurring after vaccination were summarized and analysed by Wise et al.1 The most frequently cited exposure was to recombinant hepatitis B vaccine. These authors "…hypothesized that vaccine antigens may be capable of triggering hair loss, either via telogen effluvium or through a novel autoimmune-mediated mechanism." Induction of *alopecia areata* by hepatitis B vaccine was one of their hypotheses. Based on case reports, *alopecia areata* in humans was associated in part with chronic active hepatitis,2 possibly hepatitis C virus3–5 and especially hepatitis B

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Conflict of Interest
None declared.

virus infection,6–8 and autoimmune hepatitis.9,10 However, a recent report suggested there was no relationship between human hepatitis C and *alopecia areata* 11

Alopecia areata (AA) is best defined in humans, although it occurs in many mammals as a spontaneous, reversible form of hair loss. 12–14 In humans there is a lifetime risk of 1.5–1.7% 15–17 although the frequency in other species is largely unknown. The aetiology is currently unknown but it appears to be a very complex polygenic disease.18 AA can affect any hair-bearing region of the skin and is seen in men, women, and children. Hair loss can develop in distinct patches or in diffuse areas that are most frequently on the scalp. In 7% of cases, alopecia may progress to total scalp hair loss (*alopecia totalis*) or total body hair loss (*alopecia universalis*). 16 An infectious aetiology was suspected, most recently cytomegalovirus;19,20 however, subsequent studies discounted this idea.21–25 While the aetiopathogenesis of AA remains elusive, current evidence suggests that AA may be a cell mediated autoimmune disease targeting anagen stage hair follicles.(reviewed in 26,27)

Alopecia areata presents clinically in a similar manner in dogs, horses, cattle, nonhuman primates, and rodents.12–14,28–31 The Smyth chicken is an example of a non-mammalian AA-like disease affecting feathers.32 The C3H/HeJ inbred laboratory mouse model has proven to be a useful tool for defining the genetics and mechanisms of AA and as a predictive preclinical model 13,33–35 and was accordingly adopted for the present investigation to determine if recombinant human hepatitis B vaccine induced *alopecia areata* in C3H/HeJ mice.

The study reported here found that neither recombinant human hepatitis B vaccine nor the combination of human diphtheria and tetanus toxoid induced *alopecia areata* in the inbred C3H/HeJ mouse strain that is naturally highly prone to this disease.

Materials and Methods

Mice

Due to the sexual dichotomy of frequency of occurrence of female over male C3H/HeJ mice with adult onset AA,28 only female mice were used. Two age groups were used initially, one at 8-weeks and the other at 7-months. C3H/HeJ mice (JR# 659) were obtained from The Jackson Laboratory, Bar Harbor, ME. Power calculations36 indicated that if the frequency of AA in female mice in the test group doubled from the predicted level of 20% (increase to 40%) or greater, at least 57 mice were needed to achieve a significance of $p<0.05$ (114 with controls). Sixty mice were used for vaccination and the same number for placebos to account for mice that might die due to any unanticipated reasons and aging (total of 120 per age group or 240 mice for both studies). At a disease frequency of 20%, it was expected that at least 12 mice would develop AA by 18 months of age. Due to promising results from the initial trial, the study was repeated using only 7 month-old mice divided into 3 groups of 208 each. Mice were given hepatitis B vaccine, or phosphate buffered saline, or human diphtheria and tetanus toxoid as control groups (see below).

The mice were maintained in a humidity-, temperature-, and light cycle (12:12) controlled vivarium under specific pathogen-free conditions (http://jaxmice.jax.org/html/health/quality_control.shtml#Animalhealth) 25,28 and mice housed in double-pen polycarbonate cages (330 cm^2 floor area) at a maximum capacity of four per pen. They were allowed free access to autoclaved food (NIH 31, 6% fat; LabDiet 5K52, Purina Mills, St. Louis, MO) and acidified water (pH 2.8–3.2).

Vaccination schedule

The mice were given two doses of recombinant hepatitis B vaccine (9490221, Recombivax HB®, Merck, Big Town, PA; each of 0.4 ml) subcutaneously using a 23 gauge needle,37 a dose substantially greater on a volume to body weight basis than that used in humans. The first dose was given at 8 weeks or 7 months of age, based on the age group of mice and the second dose 4 weeks later. Control mice received an equal volume of phosphate buffered saline (PBS, Sigma, St. Louis, MO). In the second study, the same dose and route of Recombivax HB® was given. As an added control, 0.2 ml of Tetanus/Diphtheria Toxoid (Product No. 49281–271-83, Aventis Pasteur Inc., Swiftwater, PA) was diluted in an equal volume of PBS and given to a third group of 208 mice. Doses were determined based on published efficacy studies using mice.38⁻42

Alopecia areata diagnosis and confirmation

A trained technician (KAS) examined all mice weekly for onset of alopecia. At the termination of the study, mice were euthanized by CO_2 asphyxiation and necropsied. Representative tissues were fixed by immersion in Fekete's acid alcohol formalin solution. After overnight fixation, tissues were transferred to 70% ethanol, processed routinely, embedded in paraffin, sectioned at 6 μm, stained with Haematoxylin and Eosin (H&E), and reviewed by a board certified veterinary pathologist familiar with this model (JPS).

Statistical analyses

Days from the date of the first injection to the date of the onset of AA were calculated. Survival analysis was performed to compare the risks of developing AA among different treatments.43 The statistical software used was JMP® 5.1 (SAS Institute Inc., Cary, NC) and R-1.9.1 (www.r-project.org).

Results

Onset and diagnosis of *alopecia areata* in mice

C3H/HeJ mice that naturally develop spontaneous AA first lose hair on their ventral abdomen, then develop patchy hair loss on the dorsal surface (Fig. 1A).28 Microscopic changes consist of infiltration in and around hair follicles with lymphocytes and are described extensively elsewhere (Fig. 1B-E).13›28 Mice with alopecia in the studies reported here had gross and histologic changes consistent with the diagnosis of AA.

Hepatitis B vaccine trial

The first trials used 2 groups of 60 mice. The 8 week old group of female C3H/HeJ mice showed no significant differences in time of onset or total numbers developing AA (Fig. 2A). By contrast, the group of 7 month old mice had a rapid onset of clinical AA shortly after receiving hepatitis B vaccine compared to those receiving PBS as a control (Fig. 2B). Though there was a slight increase in the numbers that developed AA in the hepatitis B group thirty-five weeks after receiving either recombinant hepatitis B vaccine or PBS, the difference was not significant (p=0.5689) (Fig. 2B). However, for the mice that developed AA in the 7-month old group, T-test analysis showed that the time of onset after the first injection was significantly shorter for the mice that received recombinant hepatitis B vaccine compared to those in the control group ($p = 0.0264$). One mouse (ID 127) that developed AA 9 days after injection in the PBS group was possibly an outlier from the statistical analysis. Analysis without this mouse gave a more significant result ($p = 0.0023$). These results suggested that the recombinant vaccine may initiate AA in mice and potentially humans already prone to developing the disease near the time they would naturally develop AA.

To validate these results, a second trial, using larger groups of 208 mice per group of 7-month old female C3H/HeJ mice, were treated with either recombinant hepatitis B vaccine, PBS as a negative control, or a combination of diphtheria and tetanus toxoid as a second negative control. These mice were followed for 3 months to evaluate the dichotomous change during the first two months of the initial study. While there was a slight increase in numbers of PBS controls that developed AA compared with the two vaccine groups this was not significant ($p = 0.1238$) (Fig. 2C). These results suggest that AA associated with vaccination may be within the normal background levels of the given population or premature onset of anagen secondary to wounding by the injection.

Discussion

While the aetiology of the cell mediated autoimmune disease known as *alopecia areata* is unknown, latent viral infections were suspected[3–8,19,20] but not substantiated[11,21–25] in the pathogenesis. The clinical observation that an AA-like disease, not confirmed by biopsy, followed injection of patients with recombinant human hepatitis B vaccine suggested that an epitope(s) in the vaccine might play a role.[1] The C3H/HeJ mouse model for adult onset *alopecia areata* [13,28] provided a tool to test this hypothesis, especially since this strain was used in hepatitis B vaccine efficacy trials.[37]

In the studies by Wise et al.[1] their human alopecia patient age range was 2–67 years with the vast majority being female. These results were similar to the adult onset AA mouse model in which females were more often and more severely affected than males and up to 20% of the C3H/HeJ mice develop AA by 18 months of age, equivalent to a late middle aged human.[28] This is very similar to the spontaneous adult onset forms of AA in humans.[44] In the Wise study the interval between hepatitis B vaccination and the onset of hair loss was as short as one day with 84% occurring within one month. Severe alopecia was defined as extensive hair loss over more than half the head or body and occurred in 16 of the 60 vaccinated human patients. In 16 of the 60 cases, hair loss recurred upon re-challenge with hepatitis B vaccine, 4 of which were definitive and 12 were probable. In four of the cases, hair loss recurred upon re-challenge with hepatitis B vaccination and in one case with influenza vaccine. Several cases reported associated symptoms such as arthralgia and arthritis. These changes are consistent with clinical features of human *alopecia areata* in which the disease will wax and wane. Furthermore, the secondary clinical signs in vaccination patients are suggestive of other system involvement. *Alopecia areata* patients can have concurrent autoimmune diseases including thyroiditis, systemic *lupus erythematosus*, inflammatory bowel disease, vitiligo, and other diseases (reviewed in 26). Unfortunately, the Wise study did not report histological confirmation of *alopecia areata* in the vaccinated patients.

The currently accepted concept of the pathogenesis of AA, is that it is a non-scarring, inflammatory, cell-mediated, autoimmune disease based on the characteristic peri- and intrafollicular mononuclear cell infiltrate of primarily $CD4^+$ and $CD8^+$ cells closely associated with dystrophic anagen stage hair follicles.[45,46] The underlying initiating mechanism proposed is that interferon gamma upregulates the major histocompatibility antigens, particularly MHC type I in the target regions of the hair follicle bulb.[27,47] This may also be the case in the C3H/HeJ mouse model although it is not straightforward.[48,49] It is possible that the recombinant human hepatitis B vaccine has a similar function in some patients thereby initiating AA or alopecia that has clinical features of AA. While results of experiments using relatively small numbers C3H/HeJ mice were initially suggestive, larger groups showed no effect of the vaccine, placebo, or a combination toxoid product. Statistically based genetic studies using this mouse model initially found linkage between intervals containing tumour necrosis factor alpha and its receptor (Sundberg unpublished

data) but these results disappeared when larger numbers of mice were analysed34 similar to the results obtained here indicating that large population studies are needed to confirm or refute these subtle associations. The results of this current vaccine study suggest that an AA-like sequel to human hepatitis B vaccine is probably within the background level in the general human population.

This study illustrates how rodent models prone to naturally developing complex genetic based diseases can be used in large controlled studies to investigate untoward drug or vaccine effects in other species to begin to separate out the complexities of cause and effect.

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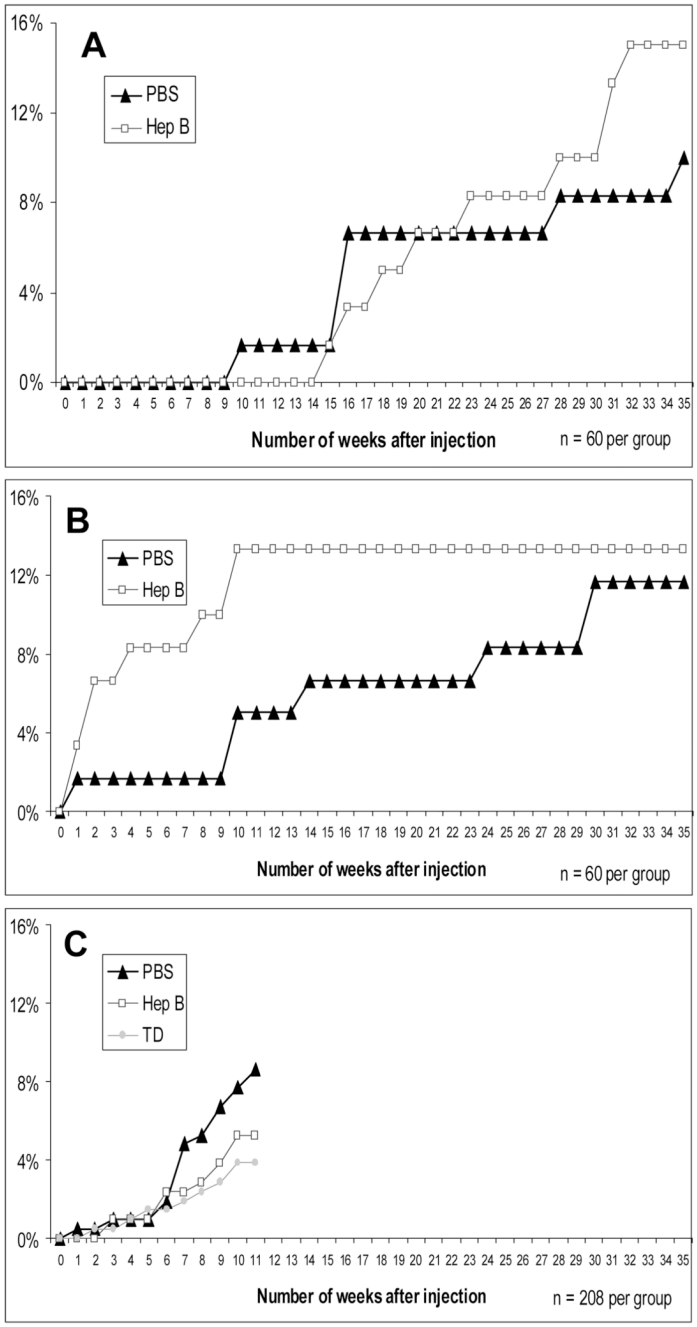

Fig. 1.

Clinical and histological appearance of *alopecia areata* in C3H/HeJ mice. Mice with *alopecia areata* developed patchy hair loss on their dorsal skin and diffuse ventral alopecia (A, lower mouse) compared to the diffuse, thick hair coat of unaffected mice (upper mouse). Microscopically *alopecia areata* affects anagen-stage hair follicles. There is a prominent infiltration consisting primarily of lymphocytes (arrows) in and around anagen hair follicles as seen in longitudinal (B,C) and oblique (D,E) sections of hair follicles. Boxed area is enlarged in right panels. Bar = 100 μm.

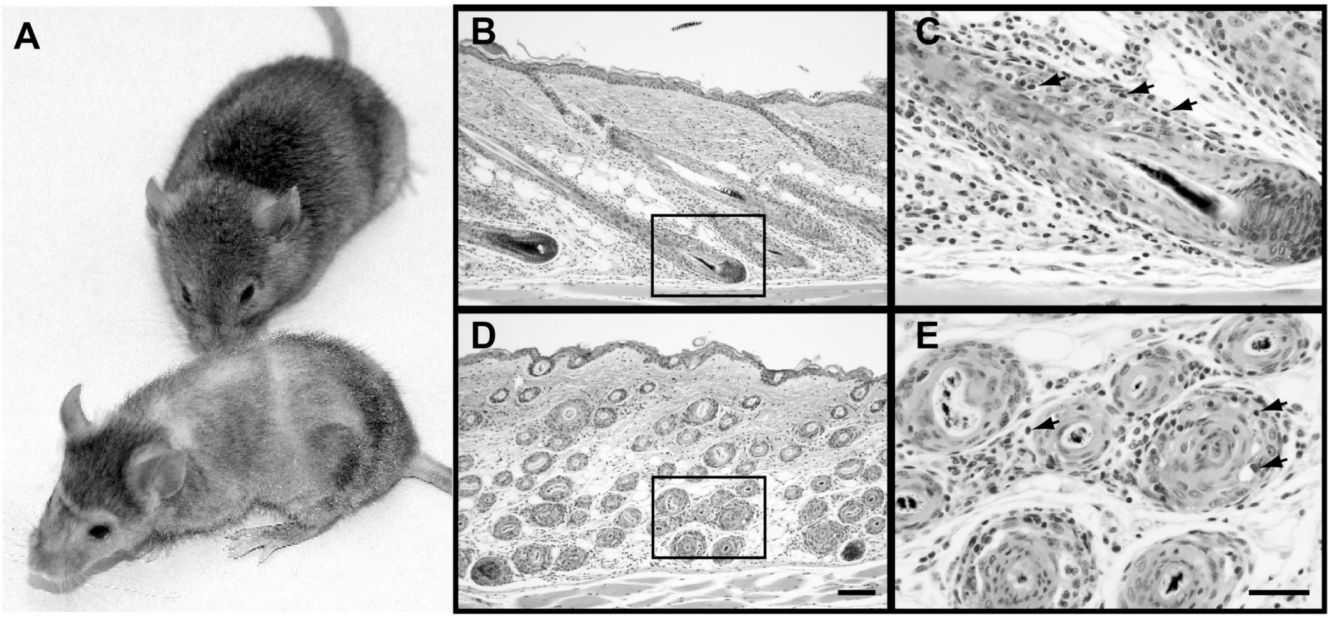

Fig. 2.

There was a slight increase in the numbers of mice that developed AA in the 8 week old group that received hepatitis B vaccine (Hep B) versus controls (PBS) thirty-five weeks after vaccination (A). This difference was not significant (p=0.5689). By contrast, 7 month old C3H/HeJ females treated in a similar manner had a rapid onset of *alopecia areata* after receiving hepatitis B vaccine compared to PBS controls (B). Time of onset after the first injection was significantly shorter for the mice that received hepatitis B vaccine compared to those in the PBS control group (p = 0.0264). In the follow-up study (C) using 3 groups of 208 female 7 month old C3H/HeJ mice there were no significant differences between the hepatitis B vaccine and PBS groups, and the third group that received doses of tetanus and diphtheria toxoid.