Androstenediol-Induced Restoration of Responsiveness to Influenza Vaccination in Mice

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Androstenediol (AED), a metabolite of dehydroepiandrosterone (DHEA), regulates innate and adaptive immune responses. To examine whether AED could effectively reverse the age-associated decline of antiviral immunity, 3-, 10-, and 22-month-old mice were treated with AED-sulfate (AED-S) for 45 days beginning 10 days prior to vaccination. Subsequently, mice were primed and boosted with suboptimal doses of a commercially-available trivalent influenza vaccine. Treatment of 10-month-old animals with AED-S during vaccination increased the titer of circulating antiviral immunoglobulin G to levels comparable with those in 3-month-old mice. Furthermore, AED-S treatment protected 10-month-old animals from intranasal challenge with a lethal dose of influenza virus 21 days after secondary vaccination. Although AED-S treatment of 22-month-old mice did not enhance vaccine responses and failed to protect against lethal challenge, the data from the 10-month-old animals suggest that treatment with AED-S will prevent the early manifestations of immunosenescence.

Aging brings with it an increased vulnerability to infectious agents. Infection is the fifth most common cause of death in the elderly population, with viral and bacterial pneumonia leading the list (1). This increased vulnerability has been linked to an age-associated deterioration of immune function, termed immunosenescence. Aged humans and rodents show declines in many aspects of protective immunity, including the formation of high-affinity antibodies, generation of long-lasting memory immune responses after vaccination, and expression of delayed-type hypersensitivity reactions to antigens initially encountered earlier in life (2–7). Although the causes of immunosenescence are not fully understood, virtually every human being who survives into advanced adulthood expresses this immunodeficient state to some extent (8–10). The challenge has been to map age-associated defects in immune responses to underlying cellular and molecular changes. This search for the underlying mechanisms is essential for the development of therapeutic strategies to enhance resistance to infectious diseases in the aging population.

One of the factors that recently has been implicated in immunosenescence is dehydroepiandrosterone (DHEA), which is the most abundant adrenal steroid in young healthy individuals. After birth, the zona reticularis of the adrenals begins to produce DHEA. Production increases throughout puberty until maximum serum concentrations are reached during the third decade of life. Then starts a slow but steady decline of approximately 2% per year in circulating blood levels, leaving a residual value of only 10%–15% during the eighth decade of life (11–12). The resulting decrease of DHEA may be partly responsible for the catabolic state that develops during aging—just the opposite that occurs during fetal development and puberty when DHEA levels are very high.

Because the decline in DHEA production parallels the onset of immunosenescence, one may hypothesize that therapeutic replacement of DHEA may restore immunocompetence to aging individuals. Indeed, Danenberg and coworkers (13–14) showed that treatment of old mice with DHEA during an influenza vaccine resulted in an increase in the humoral response and protection against subsequent influenza infection. In addition, Daynes and coworkers (15–16) reported that supplemental DHEA therapy can rapidly reverse some indicators of immunosenescence. Furthermore, DHEA has been shown to regulate the host immune response to microbial challenge because it conferred a significant protective effect against lethal viral and bacterial infections, even in young mice (17–18).

However, data suggest that metabolic conversion of DHEA to androstenediol (AED; 5-androstene-3β, 17β-diol) is necessary for regulation of immune function (19). Many of the effects attributed to DHEA that have been described in vivo, including enhancement of lymphocyte proliferation and augmentation of interleukin-2 production, are not reproducible in vitro (20–22). These functions are thought to be mediated at the cellular level after conversion to AED. This supposition is further supported by the observations that AED offers significantly better protection from infection than that conferred with DHEA (19). The enzyme 17β-hydroxysteroid dehydrogenase converts DHEA/DHEA-sulfate into AED/AED-sulfate (23). Seventy-five percent of circulating AED is produced from DHEA (24). In healthy men and premenopausal women (mean age 34.5 years), circulating serum AED levels are approximately 3.5 nmol/l (25). Although it has not been documented that metabolism to AED is necessary for the protective effect of DHEA, it has been shown that AED offered comparatively better protection against lethal infections (19), and in modulating lymphocyte (21) and macrophage cytokine production (22), than DHEA.

This study was designed to test the hypothesis that long-term treatment with AED restores immunocompetence to...
old mice. Seroconversion rates, antigen-specific immunoglobulin G (IgG) titers, and vaccine efficacy against viral challenge were studied in 3-, 10-, and 22-month-old C57BL/6 mice. The results showed that AED-sulfate (AED-S) ameliorated the age-associated decline in immunocompetence. Treatment of 10-month-old animals with AED-S during vaccination enhanced seroconversion, increased the titers of circulating antiviral IgG to levels comparable to those in 3-month-old mice, and improved resistance to infection. Furthermore, AED-S treatment, in the absence of vaccination, protected 10-month-old animals from intranasal challenge with a lethal dose of influenza A/PR8 virus. In contrast, AED-S treatment of 22-month-old mice failed to enhance their resistance to viral challenge and responses to vaccination.

MATERIALS AND METHODS

Mice

Virus-antibody-free C57BL/6NNia male mice (3, 10, and 22 months old) were obtained through Charles River, Inc. (Wilmington, MA) from the National Institute on Aging. Mice were allowed to acclimate to their surroundings for 7–10 days before initiation of experimental procedures. All mice were housed five per cage and provided unlimited access to food and water. The American Association for the Accreditation of Laboratory Animal Care–accredited facility is maintained on a 12-hour light/dark cycle (lights on at 6:00 a.m.). Prior to the experimentation, all animals were screened for antibodies to influenza virus by enzyme-linked immunosorbant assay (ELISA). Furthermore, sentinel animals were checked monthly for a variety of infectious pathogens; during the course of these experiments, all animals proved free from infection.

AED-S Treatment

AED-S (Sigma, St. Louis, MO) was supplied ad libitum in the water (25 μg/ml) beginning 10 days prior to vaccination. Water was changed twice weekly, and consumption was monitored to ensure that steroid intake was comparable among cages and age groups. AED-S was maintained in the water supply of treated mice throughout the vaccination protocol. However, 7 days prior to viral challenge, steroid-treated groups were returned to steroid-free water.

Vaccination Protocol

Mice were primed and boosted with 200 μl intramuscular injections of the commercial trivalent vaccine Fluzone (Connaught Labs, Inc, Swiftwater, PA). This whole virus vaccine contained hemagglutinin from each of the following influenza strains: A/Texas/36/91 (H1N1), A/Beijing/32/92 (H3N2), and B/Panama/45/90. Mice were primed on day 0 and boosted with a second intramuscular injection on day 21 with equivalent doses (see Figure 1).

Measurement of Fluzone-Specific Serum IgG

Mice were bled weekly, and antigen-specific IgG levels were assayed by ELISA. Polystyrene plates (Corning Glass Works, Corning, NY) were coated overnight at 4°C with 50 μl of a 1:2 dilution of stock Fluzone in bicarbonate coating buffer (pH 9.6). Plates were washed 3 times with 0.05% Tween20 in phosphate-buffered saline (PBS). Nonspecific binding was blocked at room temperature by incubating the plates for 2 hours with 100 μl of 10% goat serum in PBS. After washing 3 times, 50 μl of serial dilutions from each experimental sample were added to the plates. Plates were incubated 2 hours at 37°C and washed 3 times. Subsequently, 50 μl of horseradish peroxidase-labeled goat antimerine IgG was added, and plates were incubated for an additional 2 hours at 37°C. Finally, plates were washed 3 times, and 50 μl of substrate (ABTS [2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid]; Sigma, St. Louis, MO) was

Figure 1. Influence of aging on vaccination efficacy. Fluzone trivalent vaccine was diluted 1/200 in phosphate-buffered saline, and 200 μl was injected intramuscularly into 3-, 10-, and 22-month-old mice (n = 20 for the 3- and 10-month-old vaccinated group, n = 19 for the 22-month-old + vaccine group, and n = 15 for the control group). Animals were boosted with the same dose 21 days after primary vaccination. To determine the efficacy of the vaccination dose, mice were challenged with 40 HA U of influenza A/PR8 virus 21 days after secondary boost, and percent survival was determined.
added, and optical density was determined at 405 nm using an EL-310 ELISA reader (BioTek, Burlington, VT).

**Virus Stock**

Influenza A/Puerto Rico/8/34 (PR8) virus was obtained from the American Type Culture Collection (Rockville, MD) and propagated in the allantoic cavity of 10-day-old embryonated chicken eggs. Infectious allantoic fluid was collected, clarified by low-speed centrifugation, and stored at −70°C. The virus titer was determined to be 1280 hemagglutinating units (HAU) per ml, using human type ‘O’ erythrocytes.

**Infection of Mice**

Mice were infected with 0.05 ml of fluid containing 40 hemagglutinating units (HAU) of influenza A/PR8 virus diluted in PBS and instilled intranasally. Prior to infection, all mice were anesthetized with an intramuscular injection (0.05 ml) of 10% Rompun (Haver-Lockhart, Shawnee, KS) plus 10% Ketaset (Bristol Labs, Syracuse, NY). Serum samples were routinely screened for antibodies to influenza virus to ensure all mice were seronegative prior to experimentation.

**Statistical Analysis**

Primary research questions involved effects of categorical, experimentally-manipulated, independent variables (age, treatment with AED-S, vaccination) on both categorical (seroconversion, survival) and quantitative (lgG titers) dependent variables. Analyses of effects on categorical dependent variables were conducted using standard methods for analysis of frequency data (26). Overall effects were tested using χ², with a significant result indicating that variations in observed frequencies of the outcome variable across conditions are probably not attributable to chance. Significant effects were further examined using post hoc comparisons of pairs of groups to isolate specific group differences (26). These comparisons were conducted using simultaneous 95% confidence intervals for the difference between proportions; when such a confidence interval did not include 0, the pair of groups in that comparison could be considered as exhibiting a significant difference in proportions at the .05 significance level. The use of simultaneous confidence intervals (rather than one-at-a-time intervals) controls the overall type I error rate for these comparisons at .05. Finally, analyses of lgG titers were carried out using analysis of variance. For all results, an α level of .05 was used as the criterion for statistical significance.

**RESULTS**

### Determination of Vaccine Dose

Fluzone is a trivalent vaccine formulated for human usage. The first objective was to determine the appropriate dose for mice. The goal was to determine the lowest dose to which all 3-month-old immunocompetent mice seroconverted following a booster inoculation and that protected mice against subsequent influenza viral challenge. Five dilutions of Fluzone (1/10, 1/50, 1/100, 1/200, and 1/500) were tested (Table 1). Mice were bled 21 days after primary vaccination, and virus-specific serum IgG titers were determined. Dilutions ranging from 1/10 to 1/50 resulted in seroconversion in 100% of mice (10 of 10 mice per group). Subsequently, mice were boosted with a second dose of each dilution, and IgG titers were measured after 14 days. In addition to the lower doses, 1/100 and 1/200 dilutions induced a greater than fourfold increase in Fluzone-specific IgG in all vaccinated animals (20 of 20 mice per group). Only 65% of animals receiving the lowest tested dilution (1/500) responded to primary or secondary vaccination. To test the protective effects of vaccination in 3-month-old animals, mice were challenged intranasally with 40 HAU influenza A/PR8 virus 21 days after the second vaccination. Table 1 illustrates that a 1/200 dilution of the vaccine was the lowest dose sufficient to protect at least 80% of mice against influenza viral infection. Therefore, for subsequent experiments, the 1/200 dilution, which included 0.075 µg of hemagglutinin from each of three influenza strains (A/Texas/36/91 [H1N1], A/Beijing/32/92 [H3N2], B/Panama/45/90), was employed.

### Influence of Age on Vaccine Efficacy

The next objective was to determine how age affected the immunogenicity of the vaccine. In this experimental series, 3-, 10-, and 22-month-old C57BL/6 mice were inoculated...
with the 1/200 dilution of Fluzone and analyzed weekly for virus-specific serum IgG titers. Seventy-seven percent of the 3-month-old animals seroconverted to the primary inoculation (Table 2). In contrast, only 49% of the 10-month-old and 37% of 22-month-old animals seroconverted. Analysis of frequency of seroconversion across age groups showed significant differences ($\chi^2 = 11.97$, $df = 2$, $p = .003$). Post hoc comparisons showed significant differences in frequency of seroconversion between 3- and 10-month-old mice and between 3- and 22-month-old mice, but not between 10- and 22-month-old mice.

After 21 days, mice were subsequently boosted with a 1/200 dilution of the vaccine, and serum IgG titers were measured on a weekly basis. All 3-month-old mice showed greater than a fourfold increase in serum IgG specific for Fluzone, with a geometric mean titer of 1/23,187. Although secondary vaccination of 10-month-old mice resulted in an increase in seroconversion (69%), serum titers were significantly lower than 3-month-old mice (Table 2). The boost with Fluzone had little effect on 22-month-old animals; seroconversion was limited to 52% of the animals, and IgG titers obtained from those seropositive mice were less than 7% of those obtained from 3-month-old mice. Results of statistical tests for effects of age on seroconversion after the boost followed the same pattern as results for the primary vaccination. Age groups showed significantly different rates of seroconversion ($\chi^2 = 21.39$, $df = 2$, $p = .001$), with the 3-month-old group being significantly different from the 10- and 22-month-old groups. In addition, analysis of variance revealed a significant effect of age on levels of IgG titers ($F = 52.91$, $df = 2,73$, $p < .001$).

To examine the protective effects of the vaccine in the different age groups, all mice were challenged with a lethal dose of influenza A/PR8 virus 21 days after secondary vaccination. All but 2 of 59 unvaccinated 10- and 22-month-old mice died within 10 days of infection. Only 30% of 3-month-old unvaccinated mice survived infection (Figure 1). Vaccination with Fluzone protected 77% of 3-month-old mice from infection. Similar to the data obtained concerning seroconversion, only 29% of 10-month-old vaccinated mice survived. Furthermore, none of the vaccinated 22-month-old mice survived infection. Analysis of effects of age and vaccination on survival frequencies showed significant differences among the six experimental conditions (three age groups, two vaccination conditions), yielding $\chi^2 = 63.87$, $df = 5$, $p = .001$. Post hoc comparisons of groups showed a significant difference in frequency of survival for vaccinated versus nonvaccinated mice at age 3 months, but no such significant difference for older mice.

**Influence of AED-S on Vaccine Efficacy**

Because vaccination of the 10- and 22-month-old mice failed to protect against a lethal challenge as shown in Figure 1, we attempted to enhance the response to the influenza vaccine by treating the animals with AED-S. AED-S was supplied ad libitum in the water (25 μg/ml), beginning 10 days prior to vaccination. Although water consumption differed between age groups, the amount of drug consumed as a percentage of body weight did not differ significantly (data not shown). As before, 3-, 10-, and 22-month-old mice were inoculated with the Fluzone vaccine. All animals were analyzed weekly for Fluzone-specific serum IgG titers. AED-S was provided for the duration of the experiment. As described previously, within control groups there was a marked age-related difference in serum antibody titers. Among the 3- and 22-month-old animals, treatment with AED-S did not mediate a measurable difference in Fluzone-specific antibody titers after inoculation with the antigen. However, within the 10-month-old groups of animals, treatment with AED-S elevated serum Fluzone-specific antibodies to levels comparable with that of 3-month-old control animals and threefold higher than seen in untreated, vaccinated 10-month-old animals (Table 2). Analysis of frequency of seroconversion for the six groups (three age groups, two AED-S conditions) showed significant differences in frequencies ($\chi^2 = 56.88$, $df = 5$, $p = .001$). Post hoc comparisons showed that the effect of AED-S treatment on frequency of seroconversion was significant for 10-month-old mice, but not for 3- and 22-month-old mice. The lack of effect in the 3-month-old animals was interpreted as **Table 2. Influence of Androstenediol-Sulfate** on Vaccine Efficacy

<table>
<thead>
<tr>
<th>Age and Treatment Group</th>
<th>Seroconversion After Primary Vaccination</th>
<th>Seroconversion After Secondary Boost</th>
<th>GMT After Secondary Boost</th>
<th>% Survival After Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Month</td>
<td>27/35 (77%)</td>
<td>35/35 (100%)</td>
<td>23,187</td>
<td>77%</td>
</tr>
<tr>
<td>3-Month + AED-S</td>
<td>29/35 (83%)</td>
<td>35/35 (100%)</td>
<td>21,421</td>
<td>80%</td>
</tr>
<tr>
<td>10-Month</td>
<td>17/35 (49%)</td>
<td>24/35 (69%)</td>
<td>5,869</td>
<td>29%</td>
</tr>
<tr>
<td>10-Month + AED-S</td>
<td>23/35 (66%)</td>
<td>32/35 (91%)</td>
<td>19,740</td>
<td>66%</td>
</tr>
<tr>
<td>22-Month</td>
<td>13/35 (37%)</td>
<td>17/35 (52%)†</td>
<td>1,475</td>
<td>15%</td>
</tr>
<tr>
<td>22-Month + AED-S</td>
<td>12/35 (34%)</td>
<td>16/35 (46%)</td>
<td>1,467</td>
<td>11%</td>
</tr>
</tbody>
</table>

*Androstenediol-sulfate (AED-S) was added to the daily water supply (25 μg/ml) beginning 10 days prior to vaccination and continued until 7 days prior to viral challenge.

1Fluzone trivalent vaccine was diluted 1/200 in phosphate-buffered saline, and 200 μl was injected intramuscularly. Seroconversion after primary vaccination was determined as a four-fold increase in vaccine-specific IgG; a four-fold increase at either 7 or 14 days after injection indicated seroconversion.

2Seroconversion after secondary boost was determined both 7 and 14 days after the boost.

3The geometric mean titer (GMT) represents only those animals that seroconverted and represents the inverse of the serum dilution where detectable antibody falls below background sensitivity of the ELISA for vaccine-specific IgG.

4To determine the efficacy of the vaccine dose, mice were inoculated with 40 HAU of influenza A/PR8 21 days after secondary boost and percent survival was determined.

5Two 22-month-old vaccinated animals died prior to final virus challenge.
an inability to augment a maximal response. The lack of effect in the 22-month-old animals was interpreted as a lack of response to both vaccination and steroid treatment.

To examine AED’s ability to enhance protective immunity, 21 days after a booster inoculation, mice were challenged with 40 HAU influenza A/PR8 virus (Figure 2). In 3-month-old animals, where only 30% of unvaccinated mice survived infection, vaccination increased survival above 75%. Aging resulted in increased mortality due to infection and also reduced vaccine effectiveness. Greater than 90% of unvaccinated 10- and 22-month-old mice died within 10 days of infection. Vaccination only increased survival of approximately 30% in the 10-month-old group and did not affect survival of the older 22-month-old group. Although treatment with AED-S did not influence survival from virus challenge in the 3- or 22-month-old animals, survival of the 10-month-old animal was significantly enhanced by AED-S treatment. Forty percent of 10-month-old, unvaccinated, AED-S-treated animals survived, but 66% survived if vaccinated and treated with AED-S. This illustrates that not only was the increase in antibody titers associated with AED-S treatment efficacious but treatment of 10-month-old animals with AED-S improved the host’s resistance to lethal viral challenge, even when the treatment was stopped prior to challenge. Analysis of frequency of survival across the 12 experimental conditions (three age groups, two AED-S conditions, two vaccination conditions) showed overall significant effects ($\chi^2 = 136.15$, $d.f. = 11$, $p = .001$). Post hoc comparisons revealed that treatment with AED-S produced significantly improved survival rates for 10-month-old mice, whether or not those mice were vaccinated. No such effect was observed for 3- or 22-month-old mice.

**Discussion**

With increasing age, morbidity and mortality associated with infectious diseases rises. After the age of 65, death from pneumonia and influenza ranks as the fifth leading cause of death in the United States (1). Aging is accompanied by the progressive decline of many physiological systems, including the immune system. This decline in immune responsiveness is thought to be responsible for the increased incidence and severity of infectious diseases that occur with advancing age.

For the last century, the approach to preventing disease has been to develop vaccines that provide protective, long-lasting immunity against specific pathogens (27,28). This approach has proven effective for most immunocompetent individuals. However, aging is associated with a diminished capacity to respond to novel antigenic stimulation. As a consequence, elderly individuals respond poorly to vaccination and are left susceptible to disease. Therefore, therapeutic intervention to reverse or prevent immunosenescence would potentially lessen the significance of infectious diseases in the elderly population.

Of particular interest to these studies is the age-related decrease in the production of DHEA and AED. After peak levels are reached during puberty, there is a steady decline throughout life, leaving a residual value of only 10%–15% during the eighth decade of life (11,12). This decline is not the result of a change in the metabolism of DHEA, but instead appears to be effected by a diminished adrenal secretory rate. Adrenocorticotropic hormone challenge tests indicate that in elderly subjects, the DHEA response is decreased, unlike that of cortisol secretion, which is maintained, if not prolonged (29,30). The resulting increase in the cortisol/DHEA ratio in the blood is thought to be partly responsible for the catabolic state that develops during aging (29,30).

Therefore, because DHEA production declines progressively with age and low levels correlate with poor immune responses, several recent studies have examined whether supplementation with DHEA could reverse the age-associated decline in responses to vaccination. Even though these reports have attributed to DHEA an ability to reverse some aspects of immunosenescence (13–16,31), DHEA failed to improve responsiveness to commercially available killed trivalent vaccine in elderly human subjects (32–34). Because we have shown that AED was more potent than DHEA in augmenting immune responses in young adult mice (19), the present experiment examined whether AED could restore responsiveness to vaccination in old mice.

The data show that long-term treatment with AED-S did not effectively improve vaccine responsiveness in 22-month-old mice. Although 22-month-old mice did not re-
spond optimally to the trivalent vaccine dose, which was selected based on the response in the 3-month age group, higher vaccine doses (up to 200x) did not afford a higher level of protection to the immunosenescent 22-month-old animals (data not shown). Furthermore, in 22-month-old animals, long-term AED-S treatment did not augment vaccine efficacy of these higher doses either (data not shown). However, in 10-month-old mice that were beginning to show signs of immunosenescence (i.e., vaccine responses were diminished), AED-S treatment augmented vaccine-specific IgG titers to levels comparable with those of control 3-month-old mice. Furthermore, survival following a lethal influenza virus challenge was improved by AED-S treatment in 10-month-old vaccinated mice. The protective effect was attributed to elevated antibody titers and not directly to an immunoregulatory influence of AED during infection. This was assumed because steroid treatment was stopped 7 days prior to infectious challenge. However, even unvaccinated 10-month-old mice that were treated with AED-S had a better chance for survival from infection than did untreated mice. In fact, survival did not differ from the 3-month-old controls. These data suggest that AED played a functional role in the maintenance of an immune-competent state.

Taken together, these findings suggest that host resistance can be improved in the aging animal. Perhaps if the steady age-associated decline in DHEA and AED production was prevented, this would delay or ameliorate the age-dependent deterioration of immune function. Not only would this improve the host’s response to vaccination, but it would also aid in the immune response to pathogens for which the host was not vaccinated.

As neither DHEA (32–34) nor AED treatment improved vaccine responsiveness in the very old host, these observations suggest that immunosenescence is irreversible at some point. Perhaps the physiological pathways required to metabolize DHEA and/or AED become severely impaired with advancing age. Or perhaps, there is a concomitant loss of receptors for DHEA and AED that arise with aging. Although the data presented here do not reveal the specific mechanisms that underlie the physiology of aging, the data do suggest that treatment with AED-S will prevent the early manifestations of immunosenescence.

Acknowledgments

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