Increased propensity for amyloidogenesis in male mice

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Abstract

Background

The male sex is a risk factor for reactive amyloidogenesis in several disease entities. Environmental, socioeconomic or genetic factors may underlie this male preponderance. This study was aimed at discovering whether male sex predisposes to reactive amyloidosis also in mice and to elucidate some of the hormonal associations of this risk.

Methods

Male and female Swiss mice were subjected to an established amyloid induction protocol and the amount of their splenic amyloid was determined and compared. The effect of estrogen, progesterone, testosterone and adrenalin on amyloidogenesis was studied in both sexes by administering these hormones during amyloid induction and comparing the amount of splenic amyloid of the study mice with the control mice which received the amyloid induction protocol alone.

Results

Amyloid deposition appeared to be more abundant in male mice. This gender difference was not associated with any of the 3 sex hormones tested. Despite an expected increment, adrenalin caused an attenuation of amyloid deposition.

Conclusions

The preferential expression of reactive amyloidosis in male mice seems to be unrelated to the common sex hormones. Increased production of other hormones such as adrenalin, or perhaps an augmented susceptibility to their effect, may cause gender differences by suppressing female amyloidogenesis. Our study favors the hypothesis of genetic predisposition as the mechanism leading to sex differences in amyloidogenesis. Further validation of our findings in gonadal ablated models and other amyloid induction protocols is warranted.

Key words

Amyloid A, amyloid enhancing factor, animal model, adrenalin, sex hormones.

Introduction
Reactive amyloidosis is a condition complicating a variety of infectious, inflammatory, malignant and genetic diseases (1-4). It is defined by the deposition of amyloid A (AA) in tissues and is characterized by elevated serum levels of SAA among the other acute phase proteins (5-8).

Three lines of evidence suggest that individuals of the male sex harbor an increased risk for the development of amyloidosis. First is our experience with familial Mediterranean fever (FMF), a periodic febrile disease strongly associated with AA amyloidosis in untreated individuals. In our FMF cohort amyloidosis was more common in men (9). A similar observation has been made by others (10). Secondly, male predominance in reactive amyloidosis has been observed in several clinical entities in addition to FMF, including inflammatory bowel disease (11) and psoriasis (12). Third, in juvenile chronic arthritis (JCA), although much more common in women, the number of men and women with reactive amyloidosis is equal (13), suggesting a male preponderance in amyloidogenesis in this entity as well.

The cause of the preferential expression of reactive amyloidosis in males is unknown. Environmental and socioeconomic, as well as genetic factors, may all play roles in this finding. A controlled trial using an animal model of amyloidogenesis may shed light on this enigma.

Methods
Experimental animals
Male and female Swiss mice, 8 to 18 weeks old, originating from Survey’s Veterinary Institute, Beth Dagan, Israel, were used in the current set of experiments. The study was performed in accordance with the guidelines of the animal experimentation committee of our hospital.

Induction of amyloidosis by casein and quantification of amyloid deposition
Amyloidosis may be induced experimentally in mice by eliciting chronic inflammation using two possible approaches: (a) the classical method, in which amyloidosis is induced within 2-3 weeks (7, 15); and (b) a faster method in which amyloidogenesis is shortened to a few days. In the present study, classical amyloidosis was induced by casein according to established protocols (15, 16). Briefly, the mice were injected subcutaneously (s.c.) with 0.5 ml/day of 15% vitamin-free casein suspended in 0.02N NaOH for 5 days/week for 3 weeks. Following this treatment the animals were killed and the presence and amount of amyloid in their spleens were studied using the crush-and-smear (C&S) technique and a 5-grade semi-quantitative scale (16-19).

Enhanced induction of amyloidosis
The production of amyloid enhancing factor (AEF) and the induction of enhanced amyloidogenesis were performed as described previously (16,18, 19). Briefly, AEF was prepared from acetone-treated homogenates of spleens of pre-amyloidotic mice and injected intravenously into the studied mice (1 µg/animal). AgNO₃, an inflammation-inducing agent, was administered concurrently to the AEF-primed mice over 3 successive days (2%, 0.5 ml/day, s.c.) (14,16,18-22). Six days after the first AgNO₃ injection, the mice were killed and their spleens were examined for the presence and amount of amyloid deposition using the C&S technique. Experiments of shorter duration, lasting 2 to 4 days, which were performed to study the effect of testosterone (to be described later) before ample amyloid depo-
tion could interfere with such evaluation, were carried out as well (indicated in the text and tables). Similarly, experiments of shorter and longer duration, in addition to the standard induction protocol, were used to learn the kinetics of amyloid deposition.

**Administration of female sex hormones**

The possible effect of estrogen and progesterone on amyloidogenesis was studied as described (14). Briefly, 0.5 mg/day water soluble 17β-estradiol, or 1 mg/day water soluble progesterone, or the combined hormones, were injected intraperitoneally into male mice beginning on day 0 of the amyloid induction protocol until the mice were killed. Control mice received the same induction protocol but with the solvent (cyclodextrin) alone (without sex hormones) (14).

At the termination of these experiments the amount of splenic amyloid was studied as described above. In addition, serum sex hormone levels were determined in several experiments using the immunoochemiluminiscent method or a radioimmunoassay (RIA), as published (14); these consistently showed a 10-20 fold rise in the hormone-treated animals as compared to the controls (14).

**Administration of testosterone**

Testosterone enatate (Testoviron Depot, Schering AG, Berlin, Germany) was injected intramuscularly (i.m.), 2 mg in 0.2 ml castor oil (recini oil) into female mice at the initiation of the enhanced protocol, and again after 48h (concurrently with the third AgNO₃ injection). Control animals received the same regimen but with castor oil only (without sex hormones) (14).

Control animals received the same regimen but with castor oil only (without sex hormones) (14).

**Administration of adrenalin**

Adrenalin (Teva Pharmaceutical Industries Ltd., Petah Tikva, Israel) was injected i.p. at a dose of 4 µg/0.2 ml saline into female mice. In the casein induction protocol, adrenalin was given twice a day, 5 days per week over a period of 3 weeks, while in the enhanced protocol adrenalin was administered as above, but only over 3-4 days, according to the duration of the experiment as indicated in the results. Control groups received the same regimen but with saline alone, without adrenalin. At the termination of this set of experiments, the amount of splenic amyloid was determined as described above.

**Statistical analysis**

Statistical analysis was performed using the Student t-test.

**Results**

The amount of splenic amyloid was studied in 3 different sets of experiments, performed more than one month apart, using the same batch of AEF. In female mice, the amount of amyloid was significantly less than in male mice. The difference between the sexes was significant in 2 out of the 3 experiments, and remained significant in the combined results (Table I).

**Table I. Amyloid deposition is reduced in female mice.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Gender</th>
<th>Positive mice of total</th>
<th>Mean amount of amyloid (±SD)</th>
<th>Pvalues**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>20/20</td>
<td>3.87 ± 0.39</td>
<td>0.00005</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>15/16</td>
<td>2.75 ± 1.00</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>6/6</td>
<td>3.93 ± 0.22</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>7/7</td>
<td>3.57 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>9/9</td>
<td>4.06 ± 0.21</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12/12</td>
<td>3.80 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Male</td>
<td>35/35</td>
<td>3.93 ± 0.33</td>
<td>0.000075</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>34/35</td>
<td>3.27 ± 0.86</td>
<td></td>
</tr>
</tbody>
</table>

*The experiments were performed similarly, at different points in time. All experiments were performed using the enhanced amyloid induction protocol. The duration of all experiments was 6 days.

**Pvalues were computed using the Student t test.**

While gender differences in mouse amyloidogenesis are probably unrelated to female sex steroids, the role of testosterone has never been studied before. Table IV shows that testosterone does not promote amyloid production in the enhanced model, even though shorter duration (more sensitive) protocols were applied to allow small increments to become evident (see Methods). Finally, as male mice more readily fight each other in captivity cages, it was speculated that the higher amyloidogenic susceptibility in male mice might be due to increased secretion of adrenergic hormones. However, as seen in Table V, adrenalin given to female mice was found to have more of an inhibito-
ry than a stimulatory effect on amyloidogenesis in the longer (casein) induction protocol. An inhibition trend was also observed in the 3-day shortened (AEF) protocol which, as noted in the Methods section, was carried out to increase the sensitivity of this technique.

**Discussion**

In this set of experiments, aimed at the elucidation of the existence and causes of gender differences in murine amyloidogenesis, it was found that amyloid deposition is more abundant in male mice as compared to female mice. This gender difference was not associated with either a retarded amyloid deposition in female mice, or with an inhibitory effect of female sex hormones, or an aggravating effect of testosterone.

Adrenalin, however, which was thought to be associated with increased amyloidogenesis in male mice, was unexpectedly found to attenuate amyloid deposition.

As has already been discussed by us recently, female sex hormones may either provoke or attenuate inflammation by several mechanisms (14). Yet, if administered to female mice, female sex hormones do not affect amyloidogenesis (14). Because a possible inhibitory effect of the exogenous female sex hormones on amyloidogenesis could have been concealed by the preceding activity of endogenous sex hormones, and because male mice have estrogen and progesterone receptors in various tissues (23-29), we studied the effect of these hormones as well in male mice. However, even under these circumstances neither estrogen nor progesterone administration led to a reduction in splenic amyloid, suggesting that unresponsiveness to exogenous hormones in female mice is not due to a possible preliminary effect of the endogenous female sex hormones.

The lack of a proamyloidogenic effect of testosterone displayed in this study is not surprising and is consistent with its traditional protecting role in inflammation. Thus, the anti-inflammatory qualities of testosterone are thought to play a role in female predominance in lupus (30-34), and rheumatoid arthritis (35-37), as well as in female preference in several different mouse models of inflammation, including interstitial lung disease (32), rheumatoid arthritis (38), cartilage-breakdown (39) and Sjogren-syndrome (40).

Catacholamines are strongly associated with stress, aggression, irritability and vigilance (41-44), features more typical

**Table II.** Amyloid deposition is already at a plateau level on day 6*.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Gender</th>
<th>Duration of experiment (days)</th>
<th>Positive mice of total</th>
<th>Mean amount of amyloid (± SD)</th>
<th>P-values**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>4</td>
<td>9/9</td>
<td>3.57 ± 0.41</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>4</td>
<td>10/10</td>
<td>3.68 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>5</td>
<td>8/8</td>
<td>3.76 ± 0.32</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>5</td>
<td>9/9</td>
<td>3.69 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>6</td>
<td>6/6</td>
<td>3.93 ± 0.22</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>6</td>
<td>7/7</td>
<td>3.57 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>10</td>
<td>9/9</td>
<td>3.96 ± 0.29</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>10</td>
<td>9/9</td>
<td>3.56 ± 0.44</td>
<td></td>
</tr>
</tbody>
</table>

* All experiments were performed using the enhanced amyloid induction protocol.

** P-values were computed using the Student t-test.

**Table III.** Female sex hormones do not suppress amyloidogenesis in male mice*.

<table>
<thead>
<tr>
<th>Type of experiment</th>
<th>Material injected</th>
<th>Daily amount** (intraperitoneal)</th>
<th>Positive mice of total</th>
<th>Mean amount of amyloid (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td>Estradiol (water soluble)</td>
<td>0.5 mg</td>
<td>8/8</td>
<td>3.54 ± 0.44</td>
</tr>
<tr>
<td>Study 2</td>
<td>Progesterone (water soluble)</td>
<td>1 mg</td>
<td>8/8</td>
<td>3.74 ± 0.39</td>
</tr>
<tr>
<td>Study 3</td>
<td>Estradiol + Progesterone</td>
<td>0.5 mg + 1 mg</td>
<td>8/8</td>
<td>3.80 ± 0.44</td>
</tr>
<tr>
<td>Control †</td>
<td>Cycloextrin alone</td>
<td>22 mg</td>
<td>8/8</td>
<td>3.80 ± 0.21</td>
</tr>
</tbody>
</table>

* All experiments were performed using the enhanced amyloid induction protocol with a 6-day duration.

** The amounts of hormones represent the pure hormone content. The amount of the cycloextrin is similar to the content of cycloextrin in Study 1 to 3 experiments.

† Control mice received the enhanced amyloid induction protocol with the diluent (cycloextrin) alone (without sex hormone).

†† The differences between the study and control mice were not significant (by the Student t-test).

**Table IV.** Exogenous testosterone does not promote amyloidogenesis in female mice*

<table>
<thead>
<tr>
<th>Type of experiment</th>
<th>Duration of experiment (days)</th>
<th>Material injected** (intramuscular)</th>
<th>Positive mice of total</th>
<th>Mean amount of amyloid (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td>4</td>
<td>Testosterone</td>
<td>2 mg</td>
<td>7/7</td>
</tr>
<tr>
<td>Control 1***</td>
<td>4</td>
<td>Castor oil</td>
<td>0.2 ml</td>
<td>7/7</td>
</tr>
<tr>
<td>Study 2</td>
<td>3</td>
<td>Testosterone</td>
<td>2 mg</td>
<td>6/6</td>
</tr>
<tr>
<td>Control 2</td>
<td>3</td>
<td>Castor oil</td>
<td>0.2 ml</td>
<td>6/6</td>
</tr>
<tr>
<td>Study 3</td>
<td>2</td>
<td>Testosterone</td>
<td>2 mg</td>
<td>7/12</td>
</tr>
<tr>
<td>Control 3</td>
<td>2</td>
<td>Castor oil</td>
<td>0.2 ml</td>
<td>6/12</td>
</tr>
</tbody>
</table>

* All experiments were performed using the enhanced amyloid induction protocol.

** Testosterone in castor oil (study mice) or castor oil alone (control mice) was administered in the amount indicated at day 0, and again after 48 hrs.

*** Control mice received the enhanced amyloid induction protocol with the diluent (castor oil) alone (without testosterone), in the same volume as in the study mice.

**** The differences between the study and control mice from each group were not significant (by the Student t-test).
to male rather than female mice (45, 46). It was therefore expected that male mice, clustered in a cage and undergoing daily insults (injections), would respond by secreting high levels of catecholamine, and that this response, which is known to exert and occasionally be pronounced than that in females. Horizontally, however, in female mice receiving adrenalin, splenic amyloid deposition was found to be less abundant than in the control group (Table V). It is therefore possible that the lower propensity for amyloidosis in female mice stems from a protective effect of certain hormones such as adrenalin. Adrenalin may decrease splenic amyloid deposition by non-inflammatory mechanisms to which female mice may be more susceptible, such as splenic shrinkage (51,52), leading to the redistribution of circulating blood and the depletion of white cells from the spleen (53), which in turn deprive the supply of SAA and proteolytic enzymes from the spleen, and lead to the decrement of splenic amyloid in female mice. In conclusion, in a murine model male amyloidogenesis is quantitatively more pronounced than that in females. Hormonal factors possibly implicated in these findings include catecholamines (e.g., adrenalin) that may reduce female amyloidogenesis. As per previous and the current set of experiments, estrogen, progesterone and testosterone probably contribute only negligibly to this variation. However, additional experiments using models involving gonadal ablation with and without relevant hormonal supplement, and using more sensitive amyloidogenesis protocols, are needed to validate our findings. Gender differences in amyloidogenesis in animal models are consistent with male predominance in human reactive amyloidosis and suggest that genetic more than environmental or socioeconomic factors play a major role in this phenomenon.

References

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