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Classical and alternative pathway complement activation are not required for reactive systemic AA amyloid deposition in mice

WINSTON L. HUTCHINSON,* JEFF HERBERT,* MARINA BOTTO† & MARK B. PEPYS* *Centre for Amyloidosis and Acute Phase Proteins, Department of Medicine, Royal Free and University College Medical School, London, UK and †Rheumatology Section, Faculty of Medicine, Imperial College, London, UK

SUMMARY

During induction of reactive systemic amyloid A protein (AA) amyloidosis in mice, either by chronic inflammation or by severe acute inflammation following injection of amyloid enhancing factor, the earliest deposits form in a perifollicular distribution in the spleen. Because the splenic follicular localization of immune complexes and of the scrapie agent are both complement dependent in mice, we investigated the possible complement dependence of AA amyloid deposition. In preliminary experiments, substantial depletion of circulating C3 by cobra venom factor had little effect on experimental amyloid deposition. More importantly, mice with targeted deletion of the genes for C1q or for both factor B and C2, and therefore unable to sustain activation, respectively, of either the classical complement pathway or both the classical and alternative pathways, showed amyloid deposition similar to wild type controls. Complement activation by either the classical or alternative pathways is thus not apparently necessary for the experimental induction of systemic AA amyloid in mice.

Keywords amyloidosis, cobra venom, complement, knockout, mouse

INTRODUCTION

Induction of reactive systemic, amyloid A (AA), amyloidosis[1] in mice either by repeated chronic inflammatory stimulation[2] or acutely by injection of so-called amyloid enhancing factor (AEF), followed by a single very vigorous inflammatory stimulus[3] are the best available models for human AA amyloidosis. The deposits appear first in the mouse spleen and then in the liver and eventually the kidneys.[4] In the spleen the earliest deposits are detected around and within the peripheral mantle layer of the lymphoid follicles, and they accumulate there before extending through the follicles and also outwards into the interfollicular areas of the splenic pulp. Much is now known about the protein misfolding that underlies the transformation of soluble native precursor proteins into the pathognomonic cross-β core structure shared by all amyloid fibrils.[5,6] The importance of nucleation or seeding as an initiating factor in amyloid fibrillogenesis, including possibly the mechanism by which extracts of amyloid containing tissue or isolated amyloid fibrils themselves act as AEF, are also increasingly recognized.[7–9] However, the factors responsible for the localization and tissue distribution of amyloid deposition *in vivo* remain completely obscure.

Reports of the presence both of fixed C3[10] and of B cells bearing complement receptors within lymphoid follicles[11] led to the original demonstration that complement participates in the induction especially of T-cell dependent antibody formation[12,13] and the discovery that this is largely due to a requirement for complement in the follicular dendritic localization of immune complexes *in vivo*.[14] The finding that follicular dendritic cells are essential for splenic localization and proliferation of scrapie in mice following peripheral infection[15] suggested a possible role for complement in scrapie pathogenesis, and this was lately confirmed by ourselves[16] and others[17] using both temporary complement depletion with cobra venom factor (CVF) and

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Abbreviations: AA, amyloid A protein; AEF, amyloid enhancing factor; CVF, cobra venom factor; SAP, serum amyloid P component.

Correspondence: Professor M. B. Pepys, Department of Medicine, Centre for Amyloidosis and Acute Phase Proteins, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK. E-mail: m.pepys@rfc.ucl.ac.uk

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mice with targeted deletion of the genes for various complement proteins.

The remarkably consistent splenic perifollicular distribution of the earliest deposits of experimental AA amyloidosis therefore suggested that complement might also be involved in localization to this site. Here we report investigation of this possibility using complement depletion by CVF, and also comparison of amyloid induction in wild type and complement deficient knockout mice. No reproducible significant impairment of amyloid deposition was observed regardless of major depletion of C3 by CVF or disruption of either the classical or alternative complement pathways. Thus, in contrast to both follicular localization of immune complexes and scrapie replication on follicular dendritic cells, follicular deposition of AA amyloid apparently does not require complement activation by either the classical or alternative pathways.

MATERIALS AND METHODS

Mice

CBA/Ca mice were used for the CVF experiments. Separate strains of mice, respectively, with targeted deletion of the C1q A chain gene[18] and of the complement factor B and C2 genes[19] created as previously described, were in the C57BL/6 × 129/SV background. Controls were closely matched wild type littermates. All mice were used at 6–20 weeks of age and in each experiment the groups were matched for age and sex.

Amyloid induction

Accelerated systemic AA amyloidosis was induced by intravenous injection of 0·1 ml per mouse of a fixed dilution of amyloid enhancing factor, prepared from amyloidotic mouse spleen and tested exactly as previously described[3,7] followed immediately by a single subcutaneous injection of 0·2 ml of 2% w/v aqueous silver nitrate solution. Mice were killed for evaluation of amyloid deposition 2 or 3 days afterwards, as shown in Results. In other studies, amyloid was induced by repeated subcutaneous injections of vitamin-free casein (ICN Pharmaceuticals Inc., Cleveland, OH) 10% w/v solution in 50 mM $NaHCO_3$.[20]

Amyloid evaluation

The day before mice were killed for evaluation of amyloid deposition, each animal received a single intravenous injection of 1700 Bq of ^{125}I radiolabelled isolated human serum amyloid P component (SAP) with specific activity of 15 MBq/mg. At the end of each experiment the mice were bled out under inhalant anaesthesia, and the spleens, livers and kidneys removed into 10% buffered formalin. Radioactivity retained in each organ was measured in a gamma counter and calculated as a percentage of the injected dose. In the absence of amyloid, only traces of the ^{125}I-SAP were retained in the liver and spleen, as shown in Table 1. There was no retention above background in the kidneys in any of the experiments reported here. After counting, the organs were wax embedded and sectioned at 6 μm for staining with alkaline alcoholic Congo red to detect amyloid by its pathognomonic red-green birefringence when viewed in high intensity polarized light.[21] Quantitative histological grading of amyloid was performed 'blind', as previously described[20] by two independent expert observers in each experiment. There is a close correlation between ^{125}I-SAP retention and histological grade but the tracer method is much more sensitive and more precisely quantitative for the generally small amounts of amyloid that were present in these studies.[22] Histology can provide only a semiquantitative estimate because the continuous focusing and polarization adjustment involved in scanning sections for Congo red stained amyloid preclude precise quantitative image analysis. The same grading system was used, as reported before[20] with the following splenic appearances and approximate relative amounts of amyloid shown in parentheses: grade 0, no amyloid seen; grade 1, occasional speck (1); grade 2, occasional perifollicular strand (50); grade 3, general perifollicular strands (300); grade 4, solid perifollicular deposits (1000); grade 5, solid perifollicular and interfollicular deposits (4500).

Proteins and assays

Human SAP[20] and *Naja naja* CVF[23] were isolated and purified as previously described. SAP was radioiodinated using *N*-bromosuccinimide as previously described[24] and was separated from free iodide by gel filtration on G25 Sephadex (PD10 column, Amersham Biosciences UK Ltd, Amersham, UK) in 10 mM Tris buffered 140 mM NaCl at pH 8·0. Functional integrity of the labelled protein was confirmed by demonstration of its specific calcium-dependent binding to phosphoethanolamine immobilized on carboxyhexyl Sepharose beads.[25] Mouse serum C3 concentration was determined by electroimmunoassay as previously described.[26] The specific activity of the CVF, assayed as previously described[23,26] was one anticomplementary unit (U) per μg of CVF protein.

Statistical analysis

Significant differences between amyloid deposition in pairs of experimental and control groups were sought by non-parametric Wilcoxon rank sum (Mann–Whitney) tests, which make no assumptions about the distribution of observations. Parametric one way ANOVA was also used to compare the two different knockout groups with each other and the controls in one experiment (see Table 3). Results are shown as *P*-values.

RESULTS

Amyloid deposition in mice receiving CVF

Following a single intraperitoneal injection of 5 U of CVF in otherwise untreated mice, circulating C3 is reduced to less than 5% of starting values for about 4 days.[12,23,26] However in mice receiving the profound acute inflammatory stimulus of silver nitrate injection, that is required for immediate induction of AA amyloidosis after AEF

Table 1. Amyloid deposition in mice treated with cobra venom factor

		Amyloid deposition			
		Liver		Spleen	
Treatment	Serum C3 on day 3*	^{125}I-SAP retained†	Amyloid score‡	^{125}I-SAP retained†	Amyloid score‡
None $n=3$	590 (40)	0·9 (0·2)	0	0·1 (0)	0
5 U CVF day −1 $n=5$	299 (78)	13·7 (9·5) $P=1{\cdot}0000$	2 (0–3) $P=0{\cdot}2063$	2·5 (2·2) $P=0{\cdot}6349$	3 (0–4) $P=0{\cdot}4444$
Buffer day −1 $n=5$	> 621§	15·6 (10·9)	3 (0–3)	3·7 (0·8)	4 (3–4)
20 U CVF day −1–day 3 $n=5$	77 (12)	14·9 (3·9) $P=0{\cdot}1508$	1·5 (1–2) $P=0{\cdot}07937$	2·3 (0·5) $P=0{\cdot}1349$	3 (3–3) $P=0{\cdot}04762$
Buffer day −1–day 3 $n=5$	> 621§	22·8 (8·8)	2 (2–3)	3·6 (1·6)	4 (3–5)

All mice, except those in the 'None' group above, received AEF and $AgNO_3$ on day 0 as described in Methods.
*Mean (SD), mg/l.
†Mean (SD),% injected dose of ^{125}I-labelled human SAP retained in organs at death on day 3, after intravenous tracer injection on day 2.
‡Median (range), histological grade of congophilic amyloid deposits assessed as in Methods.
§C3 values in all mice exceeded assay upper limit and were not reassayed on dilution.
P-values are for Wilcoxon rank sum (Mann–Whitney) tests between CVF-treated and corresponding buffer-treated control groups.

Table 2. Amyloid deposition in C1q knockout mice

	Amyloid deposition			
	Liver		Spleen	
Genotype	^{125}I-SAP retained*	Amyloid score†	^{125}I-SAP retained*	Amyloid score†
Wild type (day 2) $n=17$	2·8 (1·7)	1 (0–1)	0·6 (0·5)	3 (2–4)
C1q knockout (day 2) $n=15$	4·4 (2·4) $P=0{\cdot}5209$	0 (0–2) $P=0{\cdot}5711$	0·7 (0·5) $P=0{\cdot}8208$	3 (0–4) $P=0{\cdot}5384$
Wild type (day 3) $n=12$	10·3 (5·2)	1 (0–3)	2·8 (1·0)	4 (3–4)
C1q knockout (day 3) $n=13$	6·9 (5·6) $P=0{\cdot}4966$	1 (0–3) $P=0{\cdot}204$	1·1 (0·9) $P=0{\cdot}0167$	2 (0–4) $P=0{\cdot}08853$

All mice received AEF and $AgNO_3$ on day 0 as described in Methods.
*Mean (SD),% injected dose of ^{125}I-labelled human SAP retained in organs at death on day 2 or 3, after intravenous tracer injection on day 1 or 2, respectively.
†Median (range), histological grade of congophilic amyloid deposits assessed as in Methods.
P-values are for Wilcoxon rank sum (Mann–Whitney) tests between C1q knockout and corresponding wild type control groups.

treatment, the acute phase production of C3 largely overcame the C3 depleting effect. Thus mice that received this dose of CVF on day −1, before AEF and silver nitrate injection on day 0, had approximately 50% of control pretreatment C3 values when they were killed on day 3, whilst control mice receiving only saline before the amyloid induction regime had very substantially elevated C3 concentrations (Table 1). Amyloid deposition was not statistically significantly different between the two groups (Table 1). Therefore in another experiment a very much larger dose of 20 U of CVF was given daily from day −1 until day 3, and when the mice were killed on day 3, circulating C3 values were only $\sim$ 10% of pretreated controls (Table 1). Although, amyloid deposition in the CVF treated group was similar to controls that received injections of buffer alone, the semiquantitative histological assessment, but not the more precise radiolabelled SAP retention, showed marginally less spleen amyloid (Table 1).

Table 3. Amyloid deposition in C1q knockout and factor B/C2 knockout mice

	Amyloid deposition			
	Liver		Spleen	
Genotype	^{125}I-SAP retained*	Amyloid score†	^{125}I-SAP retained*	Amyloid score†
Wild type $n=20$	10·0 (6·0)	1 (0–3)	1·5 (0·4)	3 (2–3)
Factor B/C2 knockout $n=19$	9·4 (4·3) $P=0·8112$	1 (0–2) $P=0·5838$	1·5 (0·8) $P=0·7787$	3 (0–3) $P=0·9552$
C1q knockout $n=23$	7·4 (3·2) $P=0·9903$	1 (1–3) $P=0·1886$	1·5 (0·6) $P=0·9903$	3 (2–3) $P=0·7516$

All mice received AEF and $AgNO_3$ on day 0 as described in Methods.

*Mean (SD),% injected dose of ^{125}I-labelled human SAP retained in organs at death on day 3, after intravenous tracer injection on day 2.

†Median (range), histological grade of congophilic amyloid deposits assessed as in Methods.

P-values are for Wilcoxon rank sum (Mann–Whitney) tests between each knockout group and the wild type controls. One way ANOVA tests comparing radiolabelled SAP retention among all three groups gave $P=0·1567$ for the livers and $P=1·000$ for the spleens.

Amyloid deposition in C1q knockout mice

Mice with targeted deletion of the C1q A chain gene, and with no functional C1q protein, were compared with control wild type mice with respect to amyloid deposition 2 and 3 days after induction with AEF and silver nitrate. At 2 days after induction there was no difference in amyloid deposition between the groups but on day 3 the C1q knockouts had significantly less retention of radiolabelled SAP in the spleen, suggesting reduced splenic amyloid deposition, although the histological amyloid scores did not differ from the controls (Table 2). In another experiment, amyloid was induced by daily injections of casein, 5 days per week, over a 10-week period without any use of AEF. This method of amyloid induction is much slower and less consistent than the AEF plus silver nitrate model. The amyloid deposits appear at different times and usually only in some of the mice. It therefore more closely resembles the sporadic development of human reactive systemic AA amyloidosis as a complication of chronic inflammatory conditions[1] and may also be more sensitive in detecting subtle involvement of pathogenetic factors. However 3 of 10 C1q knockout had developed amyloid by 10 weeks compared to 4 of 10 wild type controls. Two of the controls that developed amyloid had heavy deposits (grade 5 in the spleen) and two had traces only (grade 2), whereas among the affected C1q knockout mice, one had a moderate amyloid load (grade 4) and two had traces (grade 2). However, this difference between the groups was not statistically significant, nor is it likely to be biologically significant. Evidently the classical complement pathway is not essential for amyloid deposition in this model.

Amyloid deposition in factor B/C2 knockout mice

Mice with combined targeted deletion of the genes for both factor B and C2, and thus unable to activate either the classical or alternative complement pathways, were compared with control wild type mice and with a group of C1q knockout mice with respect to amyloid deposition 3 days after induction with AEF and silver nitrate. In view of the substantial variance in amyloid induction and the possibly suggestive observations in the previous experiments, much larger groups of mice were used, $n=19$–23. However, there were no statistically significant differences in amyloid deposition between either of the complement knockouts and the wild type mice (Table 3).

DISCUSSION

Amyloid deposition is variable in timing and intensity in all murine models of AA amyloidosis. Although use of AEF dramatically accelerates amyloid deposition and ensures that it occurs in almost all treated animals, the degree of variation still commands use of large groups to provide rigorous observations. After the preliminary experiments with CVF, all the studies in knockout mice therefore comprised substantial numbers of animals, and showed quite robustly that neither the classical nor the alternative complement pathway were essential for amyloid deposition.

Even very large daily doses of CVF were unable to completely overcome the profound increase in C3 production elicited by the strong inflammatory stimulus of silver nitrate injection. The CVF studies therefore could not be conclusive about a requirement for C3. However, in the absence of C1q, C3 cannot be activated via the classical pathway, and in the combined absence of factor B and C2 neither the alternative pathway and the 'tick over' amplification loop of C3 activation, nor the classical pathway, can function. The studies in knockout mice thus provide compelling evidence that neither classical nor alternative pathway complement activation is required for AA amyloid deposition in these mouse models. It remains possible that AA amyloidogenesis could involve C4 activation by the lectin pathway activation, via mannan binding protein, or even direct proteolytic cleavage of C4 and/or C3. Bound C4 and/or C3 could then interact with cellular complement receptors, but further work beyond the scope of the present study will be required to address these issues.

The AEF plus silver nitrate stimulus for amyloid deposition is extremely powerful, and overcomes, for example, the delay and diminution of amyloid deposition seen in serum amyloid P component (SAP) knockout mice treated with casein alone as a chronic inflammatory stimulus.[20] It was therefore important to test the more gentle casein induction regime, and the failure to observe a significant difference between C1q knockout and wild type mice confirmed the likely lack of involvement of the classical complement pathway in AA amyloidogenesis in mice. It may be relevant that although mantle layer B cells bearing complement receptors are the initial site of complement-dependent trapping of immune complexes, there is rapid transfer to, and then persistence on, follicular dendritic cells within germinal centres.[14,27,28] In contrast, despite the early peri-follicular appearance of AA amyloid deposits, the subsequent accumulation progresses outside and around the follicle, and only extends into the germinal centre at a later stage. The mechanisms underlying the particular localization of amyloid deposition thus remain unknown, and despite the tempting parallel with complement dependent follicular localization of immune complexes and the scrapie agent, neither the classical nor the alternative complement pathway is essential.

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