Increased Fibrillar β-Amyloid in Response to Human C1q Injections into Hippocampus and Cortex of APP+PS1 Transgenic Mice*

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(Accepted July 2, 2002)

INTRODUCTION

Complement proteins were initially found to be involved in Alzheimer’s disease (AD) with the discovery of their co-localization with Aβ deposits in human AD brain tissue by (1). Rogers and co-workers (2,3) showed that the complement protein C1q facilitates the fibrillogenesis of Aβ. Binding of Aβ to the A-chain of C1q occurs at the amino terminal portions of both proteins (3–5). Within the first 26 residues of the N-terminus of C1q, five amino acids are positive, whereas four N-terminal amino acids of the first 11 residues of Aβ 1–42 are negatively charged. A competition assay with other Aβ fragments (Aβ 1–28 or Aβ 1–16) blocked C1q binding to Aβ 1–42, but the Aβ 12–28 and Aβ 17–42 fragments did not block this binding, demonstrating the importance of the first 11 residues for binding (3). The presence of NaCl was shown to decrease binding, which further supported that C1q/Aβ binding was ionic (3).

Once bound to C1q, only fibrillar forms of the Aβ peptide have been shown to activate C1q and the complement cascade in vitro (4). The lack of complement activation by the nonfibrillar and shorter Aβ peptides in vitro agrees with in vivo observations that diffuse plaques in the aged and AD brain show minimal signs of inflammation, for example, activation of complement, activated microglia, reactive astrocytes, and dystrophic neurites (6,7).

The role of complement proteins in AD-associated amyloid deposition and inflammation is being explored through the use of transgenic models that overexpress mutant forms of the amyloid precursor protein (APP). The APP+PS1 transgenic mouse deposits Aβ at an
accelerated rate (8), and these mice were found to develop APP- and synaptophysin-positive dystrophic neurites and glial fibrillary acid protein (GFAP)-positive astrocytes, indicative of some sort of injury (9). At 6 months, however, despite the aforementioned signs of injury, there was no staining for the MHC-II marker for microglial activation. Exploration of inflammatory responses to beta-amyloid in these doubly transgenic mice revealed that microglial and astrocyte activation increased with increasing amyloid burden as mice age, and this activation was usually associated with deposits (10). They also showed C1q staining associated with fibrillar deposits and with microglia; however, unlike in AD, there was a lack of neurodegeneration associated with Aβ deposition.

Other models that deposit Aβ, including the PDAPP mouse (11), also lack associated neurotoxicity (12,13), despite the fact that a number of studies have shown the in vitro neurotoxicity of fibrillar Aβ 1–42 and Aβ 1–40 (14–16). The lack of neuronal loss associated with Aβ deposition in these models and the importance of complement proteins kindled our interests in the role of complement in transgenic mouse models. It has been suggested that a potential explanation for a lack of neurotoxicity is the relative complement insufficiency of the mouse strains upon which mutant human transgenes are bred (17,18). Mice, in general, have low levels of serum complement activation, in particular the current transgenic mouse models of AD. The APP+PS1 transgenic model has a background of C57BL/6J, DBA, Swiss Webster, and SJL mouse strains, which have been shown to have low levels of serum complement activity, using hemolytic lysis assays (18–20). If complement is playing a crucial role in the pathogenesis of AD, then the APP+PS1 transgenic mouse with its current background may not be a good model. It is this argument that led us to propose the breeding of the APP+PS1 mouse with BUB/BnJ mice, a strain that has been shown to have higher complement levels than other strains (19,20). We hypothesized that the presence of higher levels of complement may provide the necessary inflammatory milieu for sustained, widespread inflammation, and possibly neuronal loss, as seen in AD brain.

In addition to complement insufficiency in transgenic models of AD, mouse C1q differs from human C1q. It is predicted that murine C1q would not bind/interact with human Aβ as completely as human C1q. In fact, the ability of murine C1q versus human C1q to bind Aβ and induce complement activation was determined by using synthetic peptides of amino acids 14–26 for mouse and human C1q A-chains, and in the presence of Aβ, complement activation was measured (21). The extent of complement activation was measured by consumption of C4 in a standard CH50 assay as described previously (5). Murine C1q was unable to block Aβ-induced C4 consumption in normal human serum; likewise, in a more sensitive C1 hemolytic assay, Aβ in normal mouse serum versus normal human serum resulted in consumption of less mouse C1 than human C1, respectively (21). This was attributed to the differences in the murine C1q A-chain sequence that has two fewer positive charges than the human C1q A-chain and a reduced ionic interaction with the negatively charged N-terminus of human Aβ. Therefore, human C1q may be more effective than murine C1q in associating with human Aβ.

To examine the C1q/Aβ interaction in vivo, we injected human C1q into APP+PS1 transgenic mice and quantified its effect on the fibrillogenesis of Aβ and on inflammation and neurodegeneration specific to the treatment.

**EXPERIMENTAL PROCEDURE**

**Animal Handling and Breeding Strategy.** Procedures involving animals and their care were conducted in conformity with the NIH Guide for the Care and Use of Laboratory Animals and approved by the institutional animal care and use committee. Food and water were available ad libitum. Our experiments used doubly transgenic mice and nontransgenic mice obtained by crossing Tg2576 APPK670N,M671L transgenic mice (22) with line 5.1 PS1 M146L transgenic mice (23) as described previously (8). The resulting four genotypes from this mating are nontransgenic (NT), APP, PS1, and APP+PS1 mice. The Tg2576 mice are derived from a C57BL6/SJL X C57BL6 background, and the PS1 line 5.1 is derived from a Swiss Webster/B6D2F1 X B6D2F1 background. The progeny of APP X PS1 therefore have a mixture of these backgrounds, and we will refer to this as the standard background, or 96% BUB/BnJ. A modified strain background is used by breeding 96% BUB/NT and 96% BUB/BnJ females to produce litters containing NT and APP+PS1 mice with 96% BUB/BnJ background (50% BUB/NT and 50% BUB/APP+PS1, respectively).

**Surgical Procedure.** Mice in the experiment were an average age of 13 months. Mice were anesthetized with 3–5% isoflurane at an oxygen flow rate of 1 L/min. The stereotaxic apparatus (Dual Manipulator Lab Standard Model 51603; Stoeling, Wood Dale, IL) was set up with one manipulator arm holding a large probe holder for the drill and on the other side a standard probe holder for a 10 μL Hamilton syringe (#84852; Reno, NV) with 26-gauge needle. The mouse was placed in the apparatus, and the head was immobilized using 18-degree rat ear bars (#51611) and a rat nose bar (Rat Adapter #51621) modified for use with mice. An incision was made down the midline to expose the cranium, and holes were drilled on the right side over the cortex (AP +1.1 mm, ML +2.0 mm) and the hippocampus (AP +2.7mm, ML +2.6mm) relative to bregma. One microliter of either 0.1 μg/μL human C1q (Advanced Research Technologies, San Diego, CA) or 0.1 μg/μL vehicle (40% glycerol in 1× phosphate-buffered saline (PBS)) was injected at DV –3.0 mm rela-
tive to bregma for both cortical and hippocampal injections. The skin was closed with surgical staples; antibiotic was applied, and the mouse was awakened by breathing 100% oxygen.

Tissue Preparation. One week after the surgery mice were anesthetized with an overdose of pentobarbital (100 mg/kg, IP) and then perfused with 25 mL of 0.9% saline and 50 mL of 4% paraformaldehyde (PF). The brains were removed and immersion-fixed overnight in 4% PF and then cryoprotected overnight in 10%, 20%, and 30% sucrose in Dulbecco’s phosphate-buffered saline (DPBS). The fixed brain was then frozen and sectioned horizontally on a sliding microtome at 25 μm, and sections were collected in DPBS (containing 100 mM sodium azide), and stored at 4°C before being stained.

Histology

Congo Red. Mounted/air-dried sections were rehydrated in diH2O for a count of 10 and incubated for 20 min in alkaline sodium chloride solution (prepared just before use by adding 0.4 mL 1N NaOH solution to each 40 mL of NaCl solution). Sections were stained for 30 min in alkaline Congo Red solution (0.4 mL of 1N NaOH solution in 40 mL of Congo Red solution) that was prepared, filtered, and used within 15 min of mixing. Sections were then rinsed rapidly (10 s) in 3 changes of 100% ethanol (EtOH), cleared in xylene (10 dips), and coverslipped with DPX microscopy mountant.

Cresyl Violet. Mounted/air-dried sections were rinsed in diH2O for 10 dips, stained in filtered 0.05% cresyl violet for 5 min, destained in 30 dips acid H2O (1 drop glacial acetic acid/50 mL H2O) and rinsed in H2O for 10 dips. Sections were then dehydrated in 2 changes of 75% EtOH for 30 dips each, 1 min in 95% EtOH and 1 min in 100% EtOH. Sections were then cleared in Histoclear for 5 min in each of three changes and mounted with DPX.

Immunocytochemistry. Immunocytochemistry (ICC) was performed on floating brain sections in 24-well plates as described previously by this laboratory (8,24,25). Endogenous peroxidases in tissue was blocked by treating with 3% H2O2 and 10% methanol in DPBS for 15 min; then sections were washed in DPBS. Tissue was permeabilized for 30 min with 0.25% Triton X-100 and 100 mM lysine in 4% serum (for C1q staining, 0.1% bovine serum albumin in DPBS replaced serum as a blocking agent). Sections were washed again in DPBS before overnight incubation in appropriate primary antibody dilution (in DPBS+serum).

Sections were washed again and incubated for 2 h in secondary antibody, washed, incubated for 1 h in streptavidin-peroxidase or VECTASTAIN ABC-peroxidase (Vector Labs, Burlingame, CA). Sections were incubated for 5 min in 1.4 mM diaminobenzidine (DAB). For the peroxidase reaction, sections were incubated in 1.4 mM DAB containing 0.03% hydrogen peroxide for 5 min exactly. For microglial stains, DAB solutions also contained 12.65 mM nickelous ammonium sulfate 6-hydrate. After final washes in DPBS, sections were mounted on slides and then counterstained with Congo Red where indicated, dehydrated in ethanolamines, cleared, and coverslipped with DPX.

The beta-amyloid (Aβ) antibody used was a rabbit antihuman Aβ raised against the Aβ 1–40 peptide, but the antibody recognizes an N-terminal epitope in both Aβ 1–40 and Aβ 1–42. This antibody was used at a 1:10,000 dilution. Astroglia were visualized with rat anti–glial fibrillary acid protein, anti–GFAP (Zymed, San Francisco, CA; 1:10,000), while microglia were stained using rat monoclonal antibodies recognizing F4/80 (Serotec, Raleigh, NC; 1:300) or CD45 (Serotec, 1:3,000). The pathway of C1q diffusion was determined by staining with a rabbit anti–human C1q antibody (DAKO, Carpinteria, CA; 1:10,000).

Image Analysis of Stained Sections

Immunoreactive product was quantified in 4–8 horizontal sections per mouse with level of section matched a closely as possible between mice. Stain was measured in 4 locations, the injection site in the hippocampus and the cortex and the equivalent locations on the contralateral side. Quantification of β-amyloid, GFAP, F4/80, and CD45 immunostaining and Congo Red stain was performed by use of video-densitometric procedures, as described previously by this laboratory, using an Oncon V150 image analysis system (24). This software used hue, saturation, and intensity (HSI) to segment objects in the image filed based on thresholds established using standard slides. The standard slides had extreme values for intensity appropriate to the stain being analyzed. The hue and saturation thresholds were established such that on all slides measured were accurately identified. The same thresholds remained constant throughout the analysis session. Percent area occupied by any given marker was calculated as the percentage of the area sampled that was occupied by reaction product.

The area of damage was quantified by morphometry in cresyl violet–stained sections.

Statistics

Statistical analyses were performed using a SAS-based program for Windows, StatView (SAS Institute Inc., Copyright 1992–1998, Version 5.0.1). Data was analyzed by five-way analysis of variance (ANOVA) using the variables brain region (cortex or hippocampus), injection hemisphere (ipsilateral or contralateral), treatment (vehicle or C1q), strain (0% BUB/BnJ or 50% BUB/BnJ), and genotype (NT or APP+PS1). Because there is no staining of NT mice for Congo Red or Aβ, no data could be collected for the genotype factor, and four-way ANOVAs were used for these measures. T tests were performed where ANOVAs indicated significant overall effects to determine whether two specific means were significantly different.

RESULTS

Localization of C1q following Injection. The brains of mice injected with either vehicle or human C1q into the hippocampus and cortex were examined. Immunocytochemistry (ICC) of human C1q revealed the pattern of diffusion of C1q following injection (Fig. 1). In the hippocampus, the injection site (Fig. 1A,
arrow) was near the molecular layer of the dentate gyrus (DG), where staining was in the outer molecular layer extending into the hippocampal fissure. C1q staining on the ipsilateral side was also found outside the hippocampal pyramidal cell layer in the stratum oriens and alveus from CA3 through CA2 and partially through CA1. C1q immunostaining did not concentrate around Congo Red–positive deposits (Fig. 1A, arrowhead). The contralateral hippocampus (Fig. 1B) had a similar pattern of C1q staining to the injected side, but the intensity of stain was considerably less. The cortical injection site was located within the deeper layers of the cortex. Diffusion on the ipsilateral side appeared to be minimal and did not seem to follow a particular path. The contralateral cortex had no positive C1q staining; however staining was observed in the dorsal septum on both sides. Furthermore, non-injected mice did not display positive C1q staining. C1q staining was seen in vehicle-injected mice, possibly revealing minor cross-reactivity of the antibody with murine C1q induced by the injection paradigm (see Discussion). The staining patterns were similar to that in the C1q-injected mice, with stain on the contralateral side less than on the ipsilateral side; however, in most mice this stain was qualitatively less than in C1q-injected mice.

Morphometric Analysis of the C1q Injections Sites. Damage as a result of C1q injection was assessed by ex-

Fig. 1. Diffusion of C1q in hippocampus following C1q injection and subsequent damage. Photomicrographs depict ipsilateral (A, C) and contralateral (B, D) hemispheres. Human C1q-immunostained sections (A, B) are also stained with Congo Red, revealing compact amyloid deposits (arrowhead). Cresyl violet–stained sections (C, D) reveal damage that followed C1q injection. In panel A, the arrow indicates the injection area, while in panel C arrows indicate loss of granule cell neurons. DG, Dentate gyrus granule cell layer; ML, molecular layer; CA1, CA2, CA3, hippocampal pyramidal cell layer. The scale bar represents 0.4 mm.
amination of cells in cresyl violet–stained sections. In
Fig. 1, photomicrographs of representative sections re-
vealed destruction of granule cells of the inner blade of
the dentate gyrus in the ipsilateral hippocampus (Fig.
1C, arrows), which remained intact in the contralateral
hippocampus (Fig. 1D). Similarly, cortical damage was
found deep in the cortex associated with the site of
injection, with no damage on the contralateral side. The
area occupied by pyknotic cells was measured using
morphometry. Damage resulting from injections was
found on the ipsilateral side only (F1,220 = 66.90, P <
.0001), but the extent of the damage was not dependent
upon strain (F1,220 = 0.01, P = .94), genotype (F1,220 =
0.24, P = .63), region (F1,220 = 1.11, P = 0.29), or
treatment (F1,220 = 0.12, P = .73). Furthermore, C1q
did not produce damage on the ipsilateral hemisphere
exceeding that observed in vehicle-treated mice in
either 0% BUB/APP1 PS1 mice (Fig. 2A; P = .89) or
50% BUB/APP+PS1 mice (Fig. 2B; P = .36).

Amyloid Deposition at the C1q Injection Sites.
Congo Red staining was used to label fibrillar amyloid
deposits, and amyloid burden was measured using
image analysis. The effect of C1q on Congo Red per-
cent area was found to be dependent upon the strain of
the mice injected (Treatment × Strain, interaction
term; F1,96 = 6.00, P = .02). C1q was found to increase
Congo Red in the 50% BUB/APP+PS1 mice (Fig. 2D;
P = .01), but not in the 0% BUB/APP+PS1 mice (Fig.
2C; P = .56). This effect can be seen in Fig. 3 in the
hippocampus, for example, where C1q resulted in an
increased number and intensity of Congo Red–positive
deposits at the injection site (dashed box). A similar
effect on fibrillar deposits was seen in the cortex.

Aβ immunostaining was used to label both fibril-
ar and diffuse deposits. Quantitation of Aβ im-
imunoreaction product failed to reveal a significant
interaction between treatment and strain (F1,96 = 0.55,
P = .46); however, examination of the effect of C1q
injection in each strain independently revealed a trend
for C1q to increase Aβ (see Fig. 2F; P = .20) in 50%
BUB/APP+PS1 mice that did not exist in 0%
BUB/APP+PS1 mice (Fig. 2E; P = .98). This trend
can be seen in 50% BUB/APP+PS1 mice in the hip-
Iocampus injected with C1q (Fig. 4A, C) versus vehicle
(Fig. 4B, D). Photomicrographs at the hippocampal
injection sites revealed a laminar staining pattern in the
outer molecular layer of the dentate gyrus (Fig. 4A, B,
arrows). This staining appeared to consist mainly of
diffuse Aβ, with some darker, compact-like deposits
in the hilus (Fig. 4C, D, arrowheads) and adjacent
to the diffuse-stained molecular layers. The diffuse
staining appeared slightly increased in C1q-injected
(Fig. 4C) versus the vehicle-injected (Fig. 4D) hip-
Iocampus. Similar trends were seen at the cortical in-
jection site of C1q-injected 50% BUB/APP+PS1 mice.

Glial Activation at the C1q Injection Sites. Brain
sections were also examined for effects of C1q injec-
tion on staining for neuroglial markers of brain in-
flammation, astrocytes (GFAP), and microglia (CD45
and F4/80). There was a main effect of injection site
with increased staining on the ipsilateral side for
GFAP (F1,223 = 114.86, P < .0001), CD45 (F1,224 =
166.02, P < .0001), and F4/80 (F1,212 = 34.65, P <
.0001). Glial staining at the injection sites was in-
creased with respect to surrounding tissue with a re-
markable infiltration of CD45-positive (Fig. 5A) and
F4/80-positive microglia into the granule cell layer of
the DG. However, this effect was also observed after the injection of the vehicle, glycerol. Quantitation of the area occupied by immunoreaction product revealed no main effects of treatment on GFAP (ANOVA; \(F_{1,223} = 0.78, P = .38\)), CD45 (ANOVA; \(F_{1,224} < 0.01, P = .96\)), or F4/80 (ANOVA; \(F_{1,212} = 0.36, P = .55\)), and no main effects of strain on GFAP (ANOVA; \(F_{1,223} = 0.80, P = .37\)), CD45 (ANOVA; \(F_{1,224} = 0.16, P = .69\)), or F4/80 (ANOVA; \(F_{1,212} = 2.31, P = .13\)).

However, this analysis revealed a main effect of genotype on inflammatory markers in APP+PS1 mice versus nontransgenic mice. There was a main effect of genotype on GFAP (ANOVA; \(F_{1,223} = 4.23, P = .04\)), CD45 (ANOVA; \(F_{1,224} = 43.93, P < .0001\)), and F4/80 (ANOVA; \(F_{1,212} = 4.26, P = .04\)). When we compared NT and APP+PS1 genotypes for each of these markers in two regions, hippocampus and cortex, there was a significant increase in GFAP in hippocampus [Fig. 6A; \(P = .03\)]. We also observed an increase in CD45 microglial reactivity in the hippocampus (Fig. 6C; \(P = .01\)), as well as a significant increase in the cortex (Fig. 6D; \(P = .0001\)), whereas the F4/80 microglial marker had only a trend for increase with transgene in hippocampus (Fig. 6E; \(P = .27\)) and a significant increase in cortex (Fig. 6F; \(P = .03\)).

Representative photomicrographs in the hippocampus of 50% BUB/BnJ mice show astrocytes appear increased adjacent to deposits in APP+PS1 mice (Fig. 7B), which are darker with thicker processes than those in the nontransgenic mouse (Fig. 7A). Microglia stained
with the CD45 marker are located directly around deposits in APP+PS1 mice (Fig. 7D), with very little staining in NT mice (Fig. 7C). The F4/80 marker is slightly increased around only a subset of deposits in APP+PS1 mice (Fig. 7F), with NT mice having a similar level of staining (Fig. 7F).

**DISCUSSION**

The primary result of this work was that C1q injection into 50% BUB/APP+PS1 mice resulted in an approximately 60% increase in fibrillar Aβ, with a trend for an increase in total Aβ. It was hypothesized that injected C1q would bind to the human Aβ already expressed in the APP+PS1 transgenic mouse to increase fibrillogenesis as in vitro studies have shown (26). Our results are consistent with these findings.

On the other hand, there are other potential explanations for the effects of C1q on fibrillar Aβ observed in this experiment. C1q has been shown, in vitro, to reduce microglial phagocytosis of Aβ (2). It was suggested that C1q may promote excessive aggregation of Aβ, rendering microglia unable to phagocytose the compact deposits. Alternatively, it was also suggested there is a potential steric interference of C1q with Aβ, directly preventing microglial uptake of Aβ. Either explanation, increased fibrillogenesis or impaired clearance, or both, would result in increased accumulation of fibrillar Aβ as reported here.

The increase of fibrillar Aβ after C1q injection observed in the current experiment was observed only...
in transgenic mice on the 50% BUB/BnJ background. This suggests that endogenous complement proteins, found at lower levels in the standard strain background, may also be important participants in the enhanced deposition of fibrillar Aβ caused by C1q.

Despite the increase of fibrillar deposits after injection of C1q in the current experiment, it was determined that inflammatory markers were unaffected. Microglial activation was not responsible for the increase in fibrillar deposits. Previous literature suggests that complement plays an important role in the recruitment of microglia to the site of injury and the opsonization of foreign material for removal. The proteins activated later in the cascade are responsible for this activation of microglia. On the other hand, results described previously (27) suggest that stimulation of monocytes by C1q does not always result in induction of inflammatory mechanisms.

Furthermore, damage rendered by the injection as determined by cresyl violet morphology was responsible for an increased expression of endogenous mouse complement proteins. Complement in the brain has been shown to be up-regulated by brain injury; kainic acid (KA) lesions resulted in increased C1q and C4 mRNA (28,29) and increased C1q immunoreactivity (28,30,31). There is also evidence supporting the increased C1q synthesis in response to brain lesions as being produced locally by resident brain cells (30).

In addition, damage and altered behavior caused by various excitotoxins, including KA, have been
shown to vary dependent upon mouse strain. Mature DBA/2J mice are much more susceptible to seizure than C57BL/6J mice following subcutaneous administration of KA (32). Examples of this in transgenic mice include increased vulnerability to excitotoxic, as well as hypoxic damage, in primary neurons from transgenic PS1 mutant mice with the FVB/N background (33). Also, PDAPP mice obtained by (Swiss Webster × DBA/2J × C57BL/6J) male × (Swiss Webster × DBA) female showed increased sensitivity to peripheral domoic acid versus (Swiss Webster × DBA) control mice (Patrick May, personal communication).

Moreover, increases in C1q staining following kainic acid treatment appeared to be greater in mice on the BUB/BnJ background versus the C57BL/6J mice (31). The glycerol-containing vehicle used in the current experiment was also demonstrated to cause neuronal damage, and this damage was determined equivalent to that caused by injection of an equiosmolar solution of hypertonic NaCl (unpublished data). These results were similar to studies of intratesticular injection of glycerol in rats (34), where damage was attributed to the hyperosmolarity of the solutions injected. Vehicle injection resulted in C1q staining that

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**Fig. 7.** Effect of genotype on neuroglia in hippocampus of 50% BUB/NT (A, C, E) versus 50% BUB/APP+PS1 mice (B, D, F). Horizontal sections were immunostained for GFAP (A, B), CD45 (C, D), or F4/80 (E, F) and counterstained with Congo Red. Glial cells appear brown, while Congo Red–positive amyloid deposits appear red. The scale bar represents 0.1 mm for all panels.
was attributed to an induction of endogenous murine C1q (also detected by the human C1q antibody) in response to glycerol-induced damage. Murine C1q staining was not accompanied by any changes in Ab deposition. However, the C1q-injected mice, assumed to have a similar induction of endogenous murine C1q but also have exogenously administered human C1q, had altered deposition of Ab. We conclude that human C1q alone, or a combination of human and murine C1q, are responsible for the effects of increasing fibrillar Ab observed in our experiments, independent of any neurotoxic actions associated with the injection or the associated activation of microglia.

ACKNOWLEDGMENTS

This work was supported by AG15490 and by the Benjamin Trust. The authors thank the family of Ms. Dorothy Benjamin for her kind and generous donation to Alzheimer’s Research. Thanks to Dr. Paul Gottschall (U.S.F.) for the generous gift of the Ab antibody. M. N. G. would like to take this opportunity to express her thanks to Dr. Jean de Vellis for providing valuable postdoctoral experience. Jean was the kindest and most gracious mentor possible. I am pleased to be able to contribute to this tribute to him, however humbly.

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