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Phagocytosis and Deposition of Vascular β-amyloid in Rat Brains Injected with Alzheimer β-amyloid

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The presence of extracellular deposits of β-amyloid protein in the brain is a hallmark of Alzheimer's disease (AD). In an effort to determine the effect of amyloid in an animal model, the authors injected amyloid cores isolated from AD brains into the cortex and hippocampus of rats. Lipofuscin, a major contaminant of the plaque core preparation, was injected on the contralateral side and used as a control to induce an analagous phagocytic cell response. Rats were sacrificed 2 days, 7 days, and 1 month after injection and amyloid located by four histochemical techniques. Amyloid and lipofuscin move from the site of injection into otherwise undamaged neuropil, persist for at least 1 month and are both associated with increases in glial fibrillary acidic protein and microglia (OX-42) staining. By 1 week, many of the amyloid cores are ingested by phagocytes. Some of the β-amyloid–containing phagocytes migrate to the vessels and to the ventricles, and by 1 month, a significant amount of the amyloid is directly associated with the vessels. This suggests that phagocytic cells can internalize exogenous amyloid and attempt to clear it from the central nervous system (CNS). Therefore, the observed distribution of amyloid is not necessarily the initial site of deposition. (Am J Pathol 1992, 140:1389–1399)

Alzheimer's disease (AD) is characterized by the presence of extracellular deposits of amyloid in neuropil and vessel walls.[1,2] Observations from postmortem brain suggest that a slow, age-related accumulation of β-protein deposits exists in normal aging which is much more dramatic in AD and Down's syndrome.[3,4] This accumulation of abnormal deposits implies an inability to adequately remove amyloid. Amyloid deposits in AD brain are associated with many parameters of the inflammatory response.[5,6] The presence of activated microglia[7] suggests that they are attempting to phagocytize and clear the protein. However, based on ultrastructural observations, several investigators have recently suggested that microglia actually secrete or even synthesize β-amyloid.[8–10] For example, microglia could phagocytize degenerating neurites that contained amyloid precursor protein and process it, resulting in formation of β-amyloid.

The relationship between vascular and neuropil deposits of amyloid is a matter of speculation, with some researchers suggesting a vascular origin for neuropil deposits[1,11,12] and others postulating a neuronal origin for vascular deposits[13] or an independent origin for both.[14] The idea that deposits in AD brain might even arise from serum was proposed in 1912,[15] although cumulative evidence for and against this origin has been debated for several decades since Bouman's 1934 review of this problem.[16] However, despite almost 80 years of research, arguments from both perspectives exist, and the physiologic origin of vascular and neuropil amyloid remains controversial.[12–14] Because all arguments are derived from observations on pathologic material or on spontaneous amyloid deposition in normal aging humans or animals, there has been no direct experimental approach to study the disposition of β-amyloid *in vivo*. There is presently no animal model in which β-amyloid deposits can be induced, and there are no direct experiments addressing the ability of the brain to deal metabolically with β-amyloid deposits. Therefore, we decided to take mature AD amyloid deposits, inject them into the rat brain, and determine their fate *in vivo*.

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Methods

Plaque Core and Lipofuscin Preparations

Sodium dodecyl sulfate (SDS)-insoluble amyloid cores were isolated by sucrose density-gradient centrifugation from five AD brains using the method of Selkoe and Abraham.[17] Briefly, plaque core rich or control parietal and temporal cortex was stripped of meninges, and the gray matter was minced and suspended in five volumes of 2% SDS/0.1 mol/l, 2-mercaptoethanol (ME)/50 mmol/l Tris-HCl (pH 7.5) and incubated at room temperature for 2 hours. Tissue was homogenized with 15 strokes using a grade B pestle in a Dounce glass homogenizer. Homogenates were then heated to 100°C for 5 minutes and filtered through 250 and then 100 μM nylon mesh. After centrifugation of the suspension for 30 minutes at 300*g*, the pellet was washed three times in 0.1% SDS, 150 mmol/l NaCl, 50 mmol/l Tris (pH 7.5). The final pellet was resuspended in 1% SDS, 50 mmol/l Tris, sieved through 35 μM nylon mesh and loaded on a discontinuous sucrose gradient (1.2, 1.4, 1.6, 1.8 mol/l sucrose in 1% SDS, 50 mmol/l Tris, pH 7.5). The gradient was centrifuged at 72,000*g* in an AH629 swinging bucket rotor (Beckman, Mountain View, CA). The interfaces were assayed for plaque cores using Congo red and by anti-β immunostaining. The 1.6/1.8 and 1.4/1.6 interfaces were plaque-rich with the principal contaminant being lipofuscin.[17] Control brain interfaces contained principally lipofuscin. The best of the plaque-rich fractions were further purified by sorting on a FACSTAR PLUS cell sorter (Becton-Dickinson, Palo Alto, CA) to increase purity.[17] The final purity of the preparation used in these studies was estimated to be >95%. Plaque core and lipofuscin fractions were washed with 15, 35, 50, and 70% ethanol to sterilize and remove contaminating SDS. After washing in sterile saline, the samples were resuspended in sterile saline by vortexing or light sonication. Aliquots were diluted in 1–3 μl injection volume to approximately 300 cores or an equivalent wet weight of lipofuscin fraction (~0.1 μg protein).

Animals and Surgery

Fourteen Sprague-Dawley rats (250–300 g/body wt) were anesthetized with a mixture of acepromazine (1.875 mg), ketamine (37.5 mg), and xylazine (1.9 mg/kg IM). Using aseptic conditions, plaque cores and control fractions were then injected with a Hamilton syringe and a stereotaxic instrument (David Kopf, Tujunga, CA). The plaques were vortexed vigorously immediately before infusion (1 μl/2 min) and placed at two different depths using Paxinos coordinates[18]: 1) in the cortex, 1.8 mm medial-lateral, −2.80 mm anterior-posterior to bregma and 1.2 dorsal-ventral from the dura, 2) in the hippocampus, 3.2 mm dorsal-ventral from the dura. Lipofuscin was injected on the contralateral side at the same rate and volume to provide a matched control. Five minutes were allowed before needle removal to reduce backflow. At 2 days (n = 2), 1 week (n = 2), and 1 month (n = 10) after injection of the washed and sterilized plaque core preparation or lipofuscin, the rats were anesthetized and perfused with 0.9% saline and then 4% paraformaldehyde (PFA), and 0.05% glutaraldehyde using the pH shift method.[19] Brains were removed and postfixed with 10% sucrose and 4% PFA for 24 hours.

In two additional rats, the isolated amyloid cores were radiolabeled. Approximately 5 μg of chloramine T (1 mg/ml), 75 μl of 0.05 mol/l sodium phosphate, and 10 μl of NaI^{125} (1 mCi, Amersham) for 30 seconds at room temperature after which 1 mg/ml of sulfonyl methyl benzoate was added. The pellet was washed of free I^{125} by centrifugation and aspiration of the supernatant (1.0 ml × 10). Approximately 1 μCi of amyloid was then injected at the following coordinates (−2.12 anterior, 2.0 lateral to Bregma and 3.1 dorsal to dura) at the same rate and volume as described earlier. Rats were sacrificed 1 month after injection.

Immunohistochemistry

The general distribution of plaques (or lipofuscin) was first assessed by examining every fifth 20 μm cryostat section stained with hematoxylin for Congo red birefringence (or autofluorescent pigment), respectively. The injection sites were located and examined with hematoxylin and eosin (H&E). Sections were immunostained with anti-β-amyloid 14–24, raised to a synthetic peptide (HQKLVFFAEDV-C) from β-protein,[20] with the microglia marker, OX-42 (Serotec, Oxford England), or with anti-glial fibrillary acidic protein (GFAP, Sigma, St. Louis, MO.) and counterstained with Congo red for colocalization with amyloid. Immunolabelled sections were processed with Vectastain Elite kits as previously described.[21] Some of the sections were silver stained by the modified Bielschowsky method.[22] Selected silver and immunostained sections were counterstained with H&E.

Results

Fate of β-amyloid and Lipofuscin 1 Month After Injection

The purity of the gradient-isolated preparations was assessed to be more than 95% by determining the percentage of Congo red birefringent objects among the total number of objects visible with Congo red, phase con-

trast, or by autofluorescence.[20] Using the same fraction from age-matched control brains, a preparation containing mostly lipofuscin granules was obtained.[20] This was used as a control because it accounts for the presentation of a foreign antigen, as well as other unknown contaminants in the preparation.

The movement of β-amyloid and lipofuscin 1 month after injection was easily seen in the silver stain (Figure 1). In the cortex, lipofuscin (Figure 1A) and β-amyloid (Figure 1B) did not diffuse much more than 1.0 mm away from the needletrack (Figure 6), but persisted in the vicinity of the needle wound. The presence of lipofuscin or amyloid in the cortex was confirmed by autofluorescence and Congo red birefringence, respectively (not shown). In the hippocampus, lipofuscin (Figure 1C) and amyloid (Figure 1D) move laterally and medially away from the injection site just ventral to the hippocampal fissure. The presence of lipofuscin or amyloid in the hippocampus was confirmed by autofluorescence (Figure 1E) and Congo red birefringence (Figure 1F), respectively. The injected material could be readily located, and substantial amounts of the material persisted for at least 1 month. Often some of the amyloid would aggregate, but there were always some isolated plaque cores that retained the maltese cross configuration (Figure 1F).

Anti-β–Protein Immunostaining in the Rat Cerebral Cortex 1 Month After Injection

Injected amyloid plaques could also be detected using anti-β-protein immunostaining (Figure 2). Abundant β-protein immunoreactivity was observed in the needle track in the cortex (Figure 2A), which was stained dark purple. Where lipofuscin was injected, no β-protein immunoreactivity was apparent although phagocytic cells were observed along the needle track (Figure 2B), which contained yellow pigment. At higher magnification, some aggregation was apparent (Figure 2C) similar to the Congo red birefringence observed in the hippocampus (Figure 2F). Preincubation of adjacent sections with β-protein blocked all of the staining of the large dark aggregated profiles, consistent with results in human brain,[20] and partially blocked staining of some of the small debris (Figure 2D). Apparent residual staining appears to be due to the presence of a few bone fragments.

Astrocytic and Microglia Response to Injection

Macrophages, microglia and reactive astrocytes were concentrated along the needle track and in areas with injected material. However, the extent and persistence of this response appeared roughly comparable on the amyloid- and lipofuscin-injected sides (Figure 3). At low magnification, the GFAP immunoreactivity in the vicinity of amyloid (Figure 3A) and lipofuscin (Figure 3B) injections into the cortex reveal similar staining patterns. OX-42 was used to identify the phagocytic response to injury. Intense staining with OX-42 was observed in response to the amyloid injection (Figure 3C), as well as lipofuscin injection (Figure 3D). OX-42 immunoreactive amoeboid cells were present in the center of the lesion and were surrounded by immunoreactive-ramified cells 1 month after injection of either amyloid (Figure 3C) or lipofuscin (Figure 3D).

Phagocytic Ingestion of Amyloid

Amyloid immunoreactivity is frequently found within phagocytes (Figure 4A, B). Cells of this type were invariably immunostained by the phagocyte marker, OX-42 (Figure 3C, D). Similar phagocytes with negligible amyloid immunoreactivity are found on the contralateral, lipofuscin-injected side, and are discernible by their sharply defined phagocytic vacuoles, amoeboid morphology, and pale yellow color.

Movement of Injected Amyloid to Capillaries and Arterioles

Much of the amyloid injected into the rat brain becomes associated with blood vessels (Figure 5). At 2 days, there is essentially no β-amyloid immunoreactivity associated with vessels, but by 1 week, β-protein–laden macrophages are often juxtaposed to the outer walls of vessels (not shown). At 1 month, selected vessels in the injected area are clearly β-protein positive (Figure 5A, B). This immunoreactivity is blocked when adjacent sections are incubated with β-protein (not shown). Bielschowsky silver stain showed similar staining patterns around vessels (Figure 5C, D). β-protein (Figure 5A) or silver positive phagocytic cells are also associated with the vessels (Figure 5D). Hematoxylin and eosin counterstaining reveals that most of these vessels were capillaries, but some were arterioles.

Figure 6 depicts the fate of radiolabeled material 1 month after injection of β-amyloid-I^{125}. At the center of the injection into the cortex, dense labeling of silver grains exists (Figure 6A), whereas just rostral to the needletrack some radiolabeled material can be observed associated with a large vessel (Figure 6B). At the ventral tip of the needletrack in the hippocampus, there is an abundance of β-amyloid associated with the CA2 pyramidal neurons

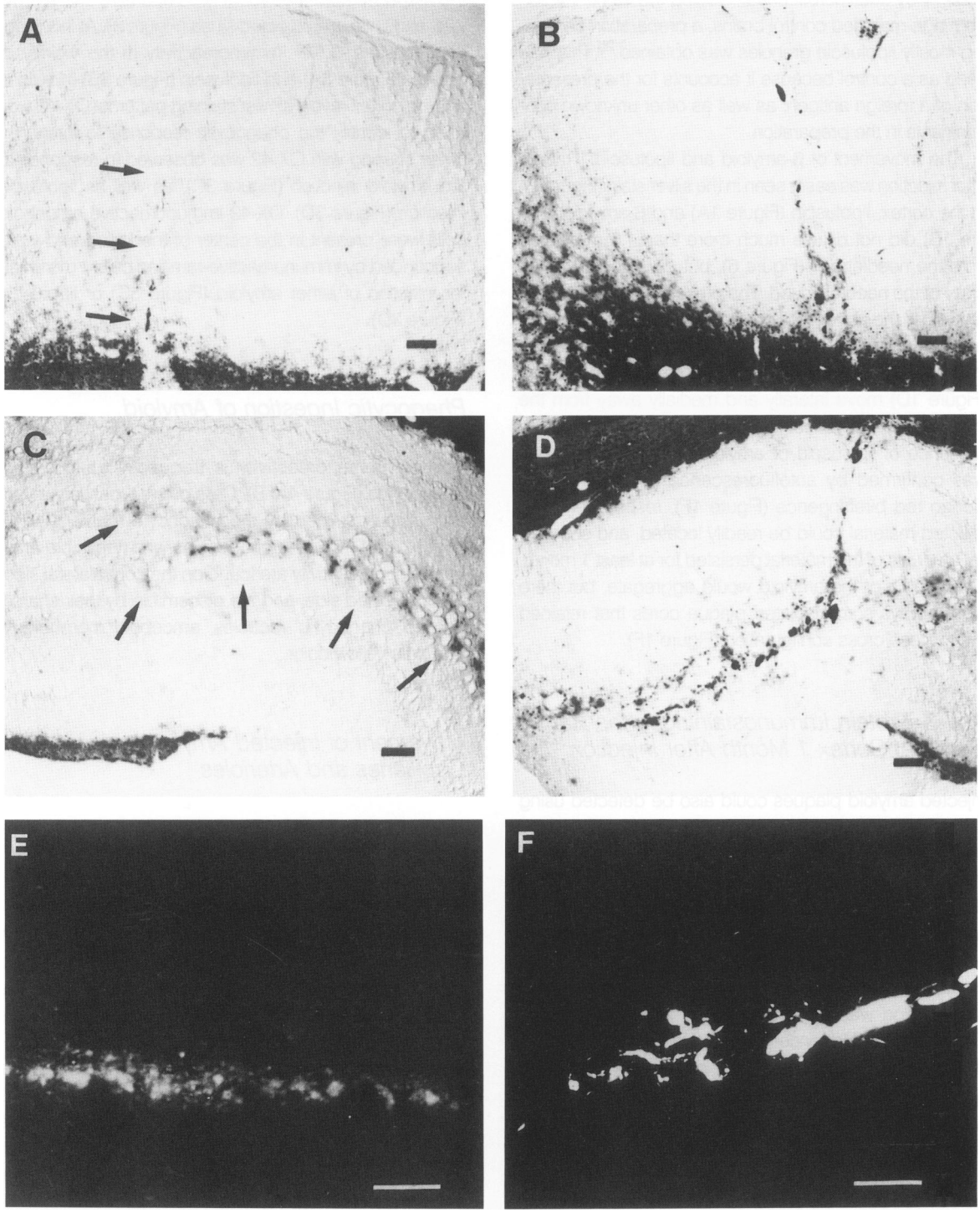

Figure 1. *Amyloid and control lipofuscin distribution in cortex and hippocampus at 1 month. Bielschowsky silver stain reveals the distribution of amyloid* **(B,D)** *along the needle track in the cortex and the lateral movement in the hippocampus 1 month after injection. The lipofuscin-injection showed a similar distribution pattern in the cortex and hippocampus as indicated by arrowheads* **(A,C)**. *Although there is variability in the 16 animals, this is the typical picture at 1 month. In the hippocampus, there is intense Congo red birefringence on the amyloid side* **(F)** *and autofluorescence on the control side* **(E)** *(bar = 100 μm.)*

(Figure 6C, E). However, 3 mm posterior to the site of injection, there has been lateral movement of amyloid which surrounds numerous vessels adjacent to the lateral ventricle (Figure 6D). Also, some amyloid becomes associated with the choroid plexus (Figure 6F).

At 1 month, β-protein immunopositive phagocytes are

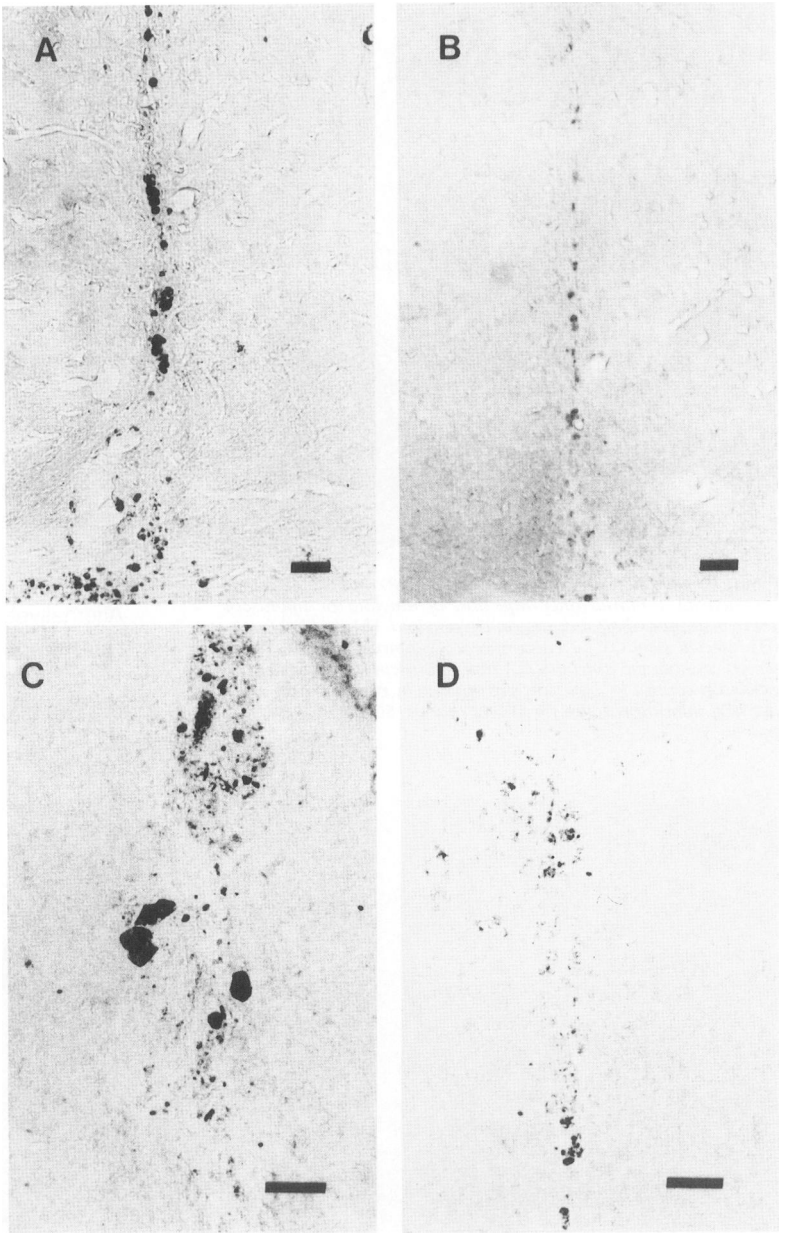

Figure 2. *Anti β-protein immunoreactivity in rat cerebral cortex 1 month after injection of amyloid plaque cores.* **A:** *β-protein immunoreactivity at the site of injection (antiserum is diluted 1:333).* **B:** *Immunoreactivity is absent on the contralateral side injected with lipofuscin.* **C:** *A higher magnification shows that some of the plaques appear to be aggregated.* **D:** *Preincubation of sections with 20 μg/ml β-protein 14-24 peptide blocks immunostaining of the large aggregated profiles and partially blocked staining of the small debris (bar = 100 μm).*

sometimes found in the hippocampus lined up along the wall of the ventricle (Figure 7A, B). Similar β-protein-containing cells could also be found in the choroid plexus when the cores moved far enough medially along the hippocampal fissure to be discharged into the ventricle (Figure 7C). Visual inspection of the sections showed no obvious reduction in β-protein immunoreactivity with time, even though by 7 days many β-protein immunoreactive phagocytes were readily apparent. Similar β-protein immunostaining is found at 1 month (and also at 2 and 3 months, not shown). We have found that in the hippocampus, injected amyloid is frequently associated with neuron loss, and in the cortex and hippocampus, injected amyloid is associated with induction of Alzheimer-related antigens.[20]

Discussion

These experiments demonstrate that when amyloid isolated from AD brain is injected into the rat brain, it can persist in neuropil for at least 1 month, can be phagocytized, and moved several millimeters to become associated with the walls of small blood vessels. The movement is particularly dramatic in the hippocampus. This distribution of plaques is surprisingly similar to the linear orientation of plaques in the inner-third molecular layer of

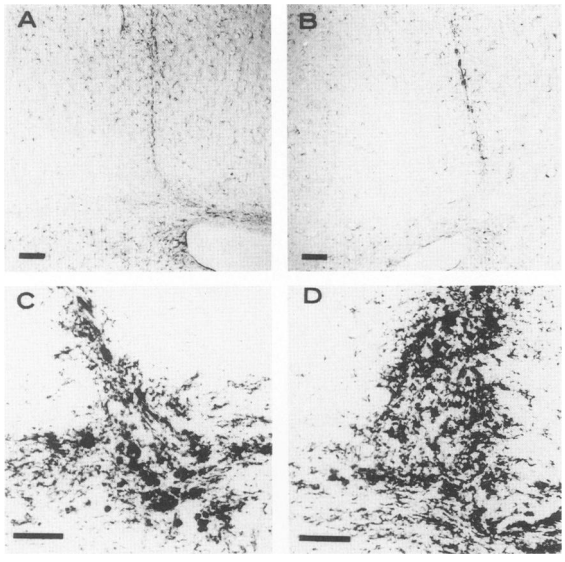

Figure 3. *Glial fibrillary acidic protein (GFAP) and OX-42 immunoreactivity 1 month after injection of amyloid or lipofuscin. There is similar GFAP staining on the amyloid-* **(A)** *and lipofuscin-* **(B)** *injected side. OX-42 immunoreactive amoeboid cells are observed on both the amyloid-* **(C)** *and lipofuscin-* **(D)** *injected sites especially central to the lesion surrounded by ramified cells which are also immunoreactive for OX-42 (bar = 50 μm).*

the dentate gyrus that is observed in human AD.[23–25] In the experimental paradigm used here, the injected amyloid cores precede the appearance of vascular amyloid deposits and accordingly appear to give rise to them. Because amyloid immunoreactivity often appears to be engulfed by amoeboid microglia and macrophages, it is tempting to hypothesize that the amyloid is carried to the microvasculature by mobile phagocytic cells. This mechanism is certainly compatible with the observation that when fluorescent microspheres are injected into the rat brain, they are rapidly ingested by amoeboid microglia, which then migrate to the vessels and meninges.[26] Similarly when microglia phagocytize cobalt-labeled axons, they migrate to the ventricles.[27] Whether brain phagocytes normally migrate or exocytose into vessels or ventricles is unclear. However, in the course of the experiments described here, occasional β-protein immunoreactive cells resembling phagocytes are found within the lumen of vessels.

Alternatively, vessel-associated phagocytes (pericytes) may engulf the amyloid, that is in the vicinity of the vessel and attempt to dispose of it through the vasculature.[28,29] *In vitro,* phagocytes have been shown to rapidly

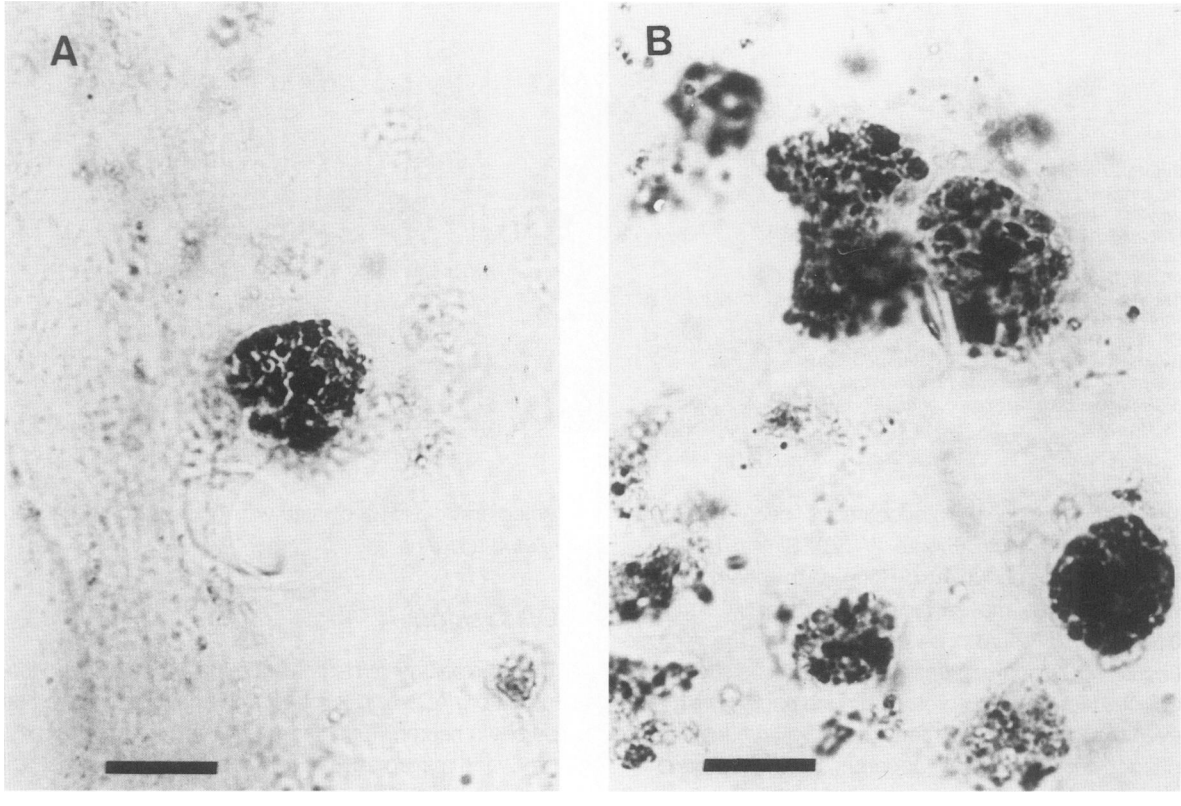

Figure 4. *β-amyloid in macrophages in the hippocampus. β-protein immunoreactivity* **(A)** *and Bielschowsky silver positive profiles* **(B)** *are present in macrophages at 1 month after injection. These cells often have a yellowish cytoplasm and the β-protein immunoreactive or silver positive material frequently appears to be in phagocytic vacuoles (bar = 10 μm).*

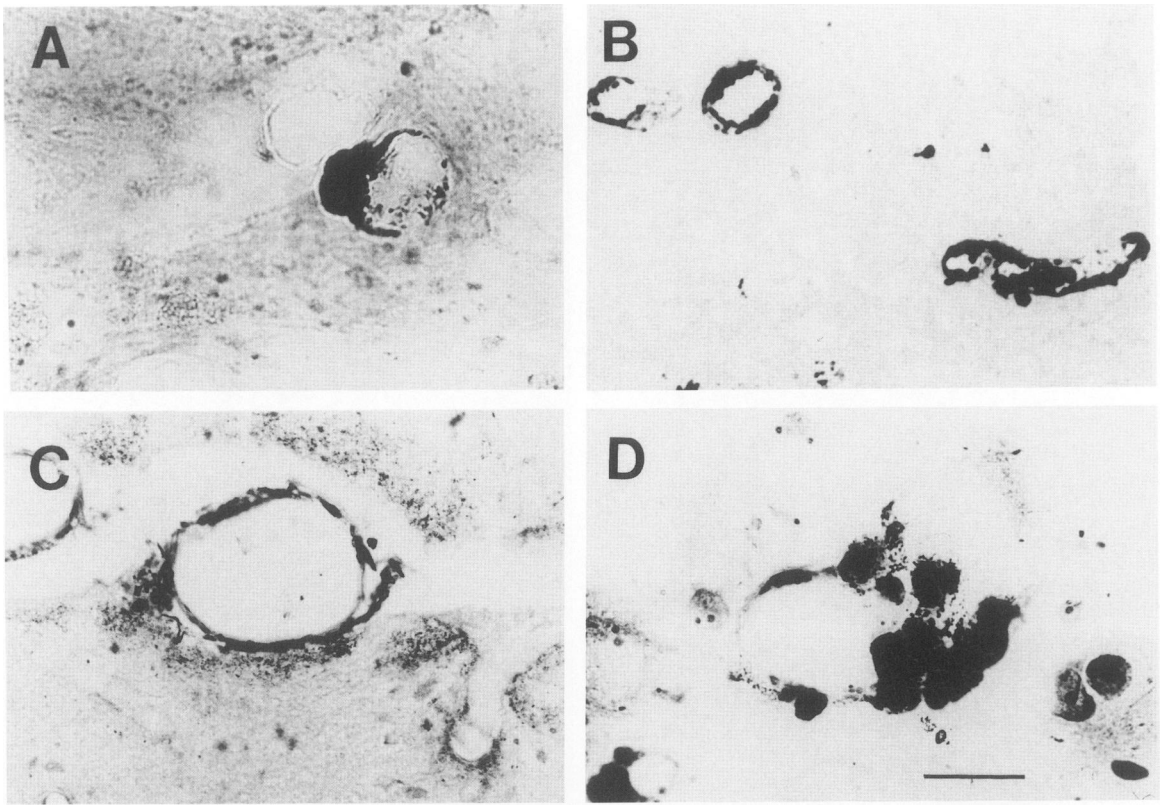

Figure 5. *β-amyloid associated with hippocampal vessels 1 month after injection. Localization of β-protein in vessels before 1 month after injection of amyloid is atypical (not shown).* **A:** *Immunohistochemical localization of β-protein is observed in a pericyte that partially surrounds a vessel. This staining is blocked by β 14-24 peptide (not shown).* **B:** *Immunohistochemical staining with anti-β-protein also reveals that β-protein can entirely surround many vessels dorsal to the hippocampal fissure.* **C,D:** *Modified Bielschowsky silver-stained sections at 1 month show a similar picture of vascular deposits (bar = 25 μm).*

exocytose previously endocytosed, undigested, exogenous radiolabelled proteins.[30] The β-protein and amyloid, thus released by phagocytic cells, may bind to the basement membrane components through heparan-sulfate proteoglycans, which are present both in the SDS-isolated amyloid[31] and in the vessel wall.

Amyloid itself or other factors contained in the injection preparation or later sequestered by the amyloid might induce the production of endogenous rat vascular amyloid. A number of observations make this unlikely. First, there is no evidence for β-amyloid deposits in the normal rat brain. Second, the time course analysis establishes that the association of large, full-fledged Congo red positive cores with vessels is only localized on the amyloid-injected side of the brain. Finally, although autoradiography of iodinated amyloid demonstrated the exogenous origin of observed deposits, the possibility of the production of endogenous rat amyloid is under investigation.

There are four major arguments supporting a vascular origin for β-amyloid in the AD brain. First, in normal aging and in specific syndromes such as the Dutch variant cerebrovascular amyloidosis, vascular amyloid can develop in the absence of significant neuropil deposits.[32] Second, amyloid is deposited in capillaries and arterial walls in AD and in many known examples of vascular amyloidosis that involve circulating amyloid precursors.[33] Third, a systemic β-amyloid deposition has been detected in skin and gut in AD that is presumably from a non-neuronal origin.[34] Even meningeal amyloid deposits are considered outside of the brain. Finally, serial sectioning of AD brain reveals that virtually every amyloid plaque contains degenerating capillaries or vessels.[35]

There is also considerable evidence supporting a neuronal origin for β-amyloid in AD brain. First, the vast majority of amyloid deposits in AD are in the brain where most of the β-amyloid precursor message is in neurons, including specific alternate transcripts.[36] Second, vascular amyloid deposits in AD are not always extensive, arguing against spillover from a vascular source. Third, cross-sectional aging studies in Down's syndrome, in which plaque and vascular amyloid deposits essentially identical to those in AD develop, show that neuropil β-protein deposits appear to develop first.[37] Fourth, the earliest deposition of amyloid appears to center on neurons and their arbor rather than vessels,[38] and capillary

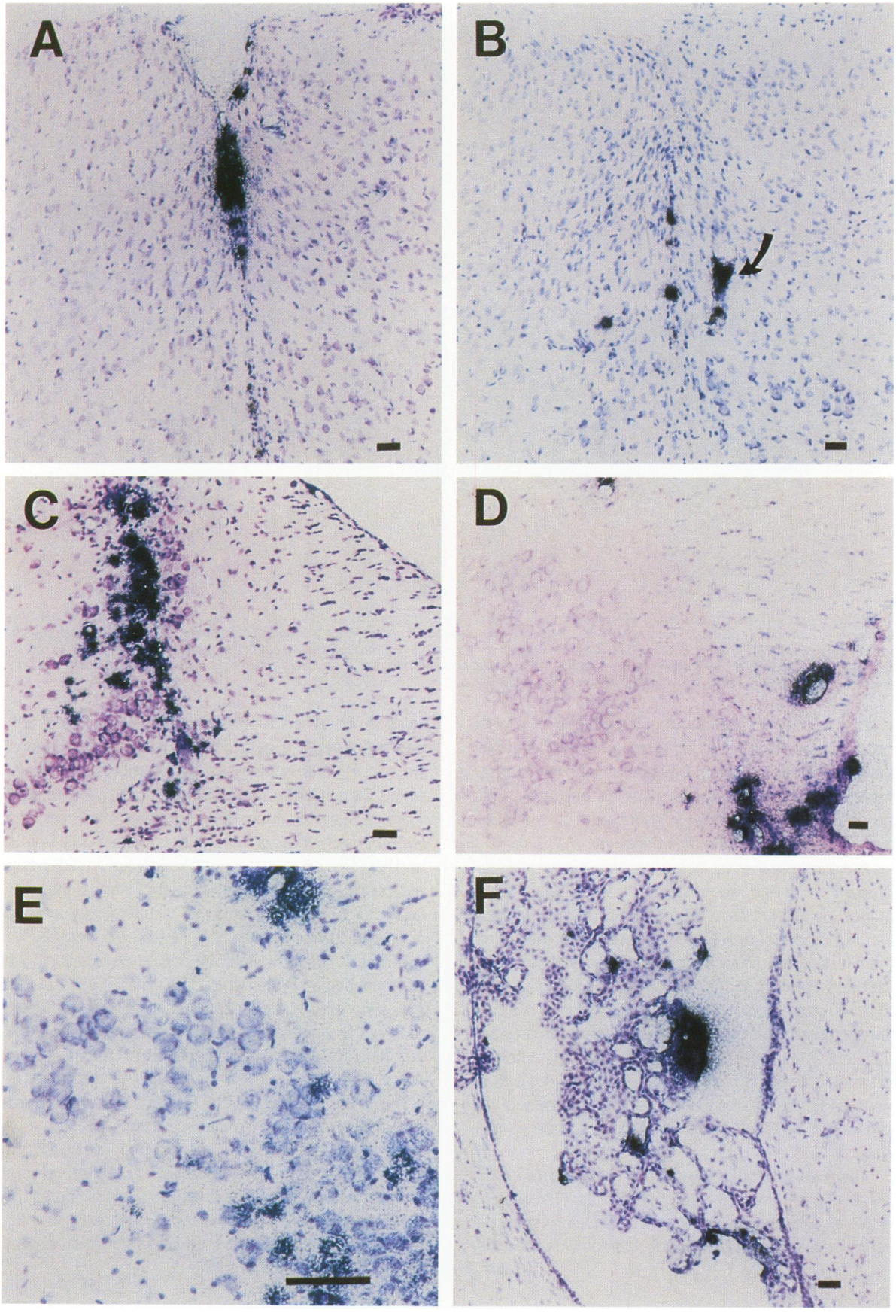
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Figure 6. *Distribution of I^{125}-labeled amyloid cores 1 month after injection. Slides were exposed to autoradiographic emulsion for 4 weeks and developed in rapid fix. They were then stained with toluidine blue* (A-F) *(and eosin,* A,D*).* A: *The high density of silver grains in the cortex at the injection site demonstrates the persistence of amyloid.* B: *Approximately 1 mm rostral to the site of injection, a few silver grains are still observed adjacent to the needle track and some amyloid has migrated to an adjoining vessel as depicted by the arrow.* C: *In the hippocampus near the ventral tip of the needletrack, amyloid is concentrated in the CA2 pyramidal neurons and a few scattered vessels.* D: *Approximately 3 mm posterior to the site of injection, there is a high density of silver grains surrounding numerous vessels and lining the ventricle.* E: *A high magnification micrograph of* (C) *demonstrates the persistent association of the amyloid with the CA2 pyramidal neurons near the site of injection.* F: *This micrograph demonstrates that some of the amyloid has been cleared to the choroid plexus. Bar = 25 μm.*

involvement may simply be a necessary result of the size of plaques and high cerebral capillary density.

The data presented here suggest a novel pathway for a transition from neuropil deposits to vascular deposits. Amyloid in the AD brain is often associated with activated microglia[7,39] and amyloid fibrils have been repeatedly observed apparently within these microglia.[8,29,40–42] These findings are thus consistent with the possibility that microglia normally engulf amyloid in AD. However, the detection of amyloid fibrils or β-protein immunostaining in the phagocytic vacuoles of microglia/macrophages in AD is uncommon. When observed, recent investigators suggested that the activated microglia surrounding amyloid cores are synthesizing and secreting amyloid.[8,9] Our results would support the more conservative interpretation that these phagocytes ingest, rather than synthesize, a precursor substrate and then may exocytose amyloid deposits to the vasculature.[29] This view is supported by the observation that in the dog, monkey, and man, the macrophages and microglia that are associated with cellular debris are closely apposed to and contain amyloid deposits.[29,41,42] Moreover, microglia-containing antipaired helical filament immunoreactivity have been found in plaques and vessels of the AD brain, the macrophages of the leptomeninges, and cerebrospinal fluid,[43] suggesting that the material of neuronal origin is phagocytized in the neuropil and can travel with the phagocytes to the microvasculature.

Finally, uptake of antigen by macrophages and microglia is considered to be an essential, antigen-presenting step required for mounting an immune response.[26] Therefore, indirect evidence that β-protein deposits are ingested by microglia or macrophage phagocytes in AD is provided by the detection of anti-β-protein antibodies in the cerebrospinal fluid of AD patients.[44] The fact that substantial amyloid deposits accumulate in humans would thus imply the existence of an inefficient removal process. The present experiments support the concept that preformed amyloid fibrils are ingested by brain macrophages in the rat and raise the possibility that the same phenomenon can occur in humans. If this is the case, then the wide spectrum of amyloid deposits that are found in neuropil, white matter, and vessels[45] need not necessarily reflect the initial site of deposit. At least some vascular deposits may derive from deposition by brain phagocytes attempting to reduce the amyloid burden in the neuropil. In Down's syndrome (DS), amyloid deposits are reported to develop in the neuropil before vascular amyloid can be detected.[4,37,46] Accordingly, the present study offers a potential mechanism for transport to the vessels supported by the results of the injection of radiolabeled β-amyloid and immunocytochemical detection of β-amyloid. Of course, there may also be independent vascular sources of amyloid.

The efficacy of phagocytes in removing human amyloid deposits in rat brain was not quantitatively investigated in this study, but the persistence of large amounts of Congo red, silver, and β-protein– positive material after 1 month (and even several months, not shown) suggests that the process is slow. Furthermore, the amyloid immunopositive brain macrophages were not dramatically reduced in number or intensity of staining with time, suggesting that phagocytosis was not necessarily accompanied by successful digestion. The persistence of amyloid

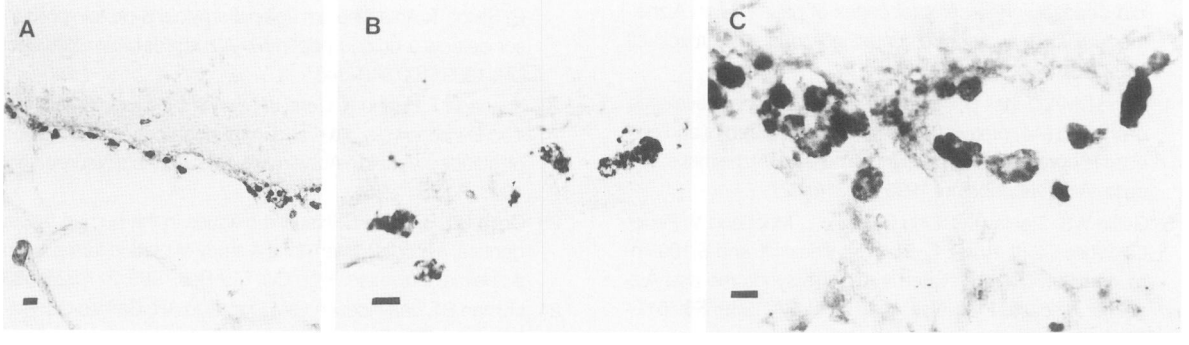

Figure 7. *β-amyloid staining of rat brains. At 1 month, amyloid β-protein immunoreactive phagocytes are found along the wall of the ventricle. At 1 month* (A) *or 1 week* (B) *after injection.* C: *In some instances injected cores are also found in the choroid plexus 1 week after injection (bar = 10 μm).*

was also supported by the results of injecting radiolabeled amyloid. In contrast, injection of the 1 μCi of radiolabeled basic FGF showed a dramatic decrease in silver grain density 7 days after injection.[47]

The rat mounts a vigorous attempt to remove fully developed human amyloid cores. In fact, the attempted clearance of β-amyloid from the rat brain is similar to that of other foreign proteins including lipofuscin in our model. This raises the possibility in humans that the site of β-amyloid deposits observed at autopsy is not necessarily the site of initial deposition. The observation that phagocytic cells can move or remove amyloid is supported by the recent ultrastructural observation that in aged squirrel monkeys with cerebrovascular amyloidosis, lipid-laden macrophages often surround amyloidotic vessels,[48] and in AD brain, amyloid is ingested by invading macrophages in the area of recently infarcted tissue.[49]

Acknowledgments

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