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13. ABSTRACT ***(Maximum 200 Words)*** The role of mitochondrial injury and subsequent apoptosis in the process of neurodegeneration is gaining increasing attention and importance. The animal model system utilized in this project is the New Zealand white rabbit which is treated with an aluminum compound (Al maltolate)by direct injection into the brain cisterna. Early studies have shown that this treatment induces oxidative injury, presumably to mitochondria. Our early work suggested that the aging process increased susceptibility to this Al-induced injury, as we have now confirmed by isolating brain tissue and obtaining cell fractions. We have demonstrated that the release of Cytochrome C through a megachannel in mitochondria, termed the mitochondrial permeability transition pore (MTP), is present in aged rabbits but not in young adults. By direct injection of Cyclosporin A into the brain cisterna of these experimental animals, we have shown that we can block the opening of this megachannel, and have observed blocking of the release of Cytochrome C from mitochondria into the cytosol. Young adult animals appear to be protected from this mitochonrial injury, but as yet we do not know by which mechanism. Cytochrome C release through the MTP is known to be the initial step in activating caspases, and eventually in leading to apoptosis and death of the neuron. Our initial work has centered around optimizing the dose of Al maltolate and establishing that we can indeed inject Cyclosporin A directly, without apparent clinical problems. We have developed techniques for obtaining cell fractions which are providing us with important new information. We are employing immunohistochemistry as a confirmatory technique, although we anticipate that this approach will play an increasingly important role for our future studies. We have established almost all of our techniques and so far have performed studies on 67 rabbits.

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John Saw 7/27/00

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INTRODUCTION:

The purpose of this work is to determine the role of mitochondrial injury in neurodegeneration, particularly the increased susceptibility to injury during aging. The initial hypothesis proposed was that oxidative stress induced by intracisternal aluminum administration to rabbits induces opening of the mitochondrial permeability transition pore (MTP), resulting in programmed cell death or apoptosis. Control of these events is known to be via the apoptosis regulatory proteins, Bcl-2 and Bax. We proposed that assessment of the role of the MTP opening would be accomplished by blocking this event with Cyclosporin A, and that changes in susceptibility during the aging process would be evaluated by comparing the effects of aluminum administration to both young adult and aged rabbits.

Progress in understanding the factors controlling apoptosis is being made at a rapid rate, and new information has allowed us to use markers for this process that were not clearly defined when our proposal was submitted in the autumn of 1998. The reviewers of our proposal suggested that caspase activation and other markers for programmed cell death should be applied in our experiments. Our work over the first 12 months incorporates these suggestions and other new information that has appeared recently in the literature. Presented here are details of our investigations which really got under way in September, 1999, when the research staff was recruited.

BODY:

Task 1: Optimization of the aluminum maltolate dose.

According to our Statement of Work for the first 12 months, we sacrificed 6 young (~ 8 - 12 months) and 6 aged (3-5 years) female New Zealand white rabbits and compared the brain weights with body weight. The results are summarized as follows in Table 1:

Table 1: Brain and body weights of young and aged rabbits: initial study.

Young		**Aged**	
Brain weight (g)	**Body weight** (kg)	**Brain weight** (g)	**Body weight** (kg)
9.6	3.8	9.8	4.9
9.4	3.6	9.8	4.8
9.8	3.8	9.0	5.4
8.3	2.4	9.5	4.7
8.5	2.4	10.8	5.3
9.0	2.4	9.9	4.8
Mean 9.1	**Mean** 3.1	**Mean** 9.8	**Mean** 5.0
SD ± 0.61	**SD** ± 0.73	**SD** ± 9.8	**SD** ± 0.29

Although there is only an approximately 10% difference in brain weight between the aged and young animals, there is a ~ 60% increase in body weight in the older age group. We consider the difference in brain weight to be negligible, especially since the intracisternal injection procedure does not allow for the injection of an exact volume (tuberculin syringes are employed, and an air bubble is introduced in order to

visualize the demarcation between the injection solution and the cerebrospinal fluid). Thus, in subsequent experiments, we have proceeded to inject the same quantity of solution into the aged and young animals. We have continued to monitor brain and body weights for all animals used so far, and these weights are summarized in Table 2.

Table 2: Brain and body weights of young and aged rabbits: composite data.

	Brain weight (g)	Body weight (kg)
Young (n=32)	9.10 ± 0.33	2.84 ± 0.30
Aged (n=35)	10.43 ± 0.44	5.29 ± 0.58

Using this information on rabbit brain weights, we have chosen an aluminum (Al) maltolate dose of 2.5 μmole (100 μL of a 25mM solution) for the initial experiments. This dose was administered to both young and aged animals which were sacrificed 72 hours after the injection. All procedures were carried out as described in the attached manuscript (Appendix 1) which has been submitted for publication. The results of this study are detailed as follows:

Microscopic evaluation of cortical-hippocampal morphology in animals treated with Al maltolate, Cyclosporin A and/or saline.

Fourteen μm-thick frozen sections were stained with the Bielschowsky's silver impregnation method for axons (Appendix 2, page 806).

Group 1: Two young rabbits treated intracisternally with 2.5 μmole Al maltolate and sacrificed after 5 days:

Both animals exhibited definite motor deficits before sacrifice. In both animals, the frontal and temporal cortex display numerous silver-positive neurofilamentous aggregates ("neurofibrillary tangles"), which are also visible in the striatum. The hippocampus also is involved, but to a lesser extent than the cortical and striatal regions.

Group 2: Four aged rabbits treated intracisternally with 2.5 μmole Al maltolate and sacrificed after 72 hours: (brain areas similar to those in Group 1 were examined).

Rabbit 1: Prominent neuronal filament arrays, but no definite neurofibrillary tangles. No tangles are found in the hippocampus or thalamus.

Rabbit 2: No neurofibrillary tangles.

Rabbit 3: No neurofibrillary tangles.

Rabbit 4: Some subtle coalescence of neurofibrils in neurons of the ventral thalamic region; nothing evident in the frontal or temporal cortex or hippocampus.

Group 2: Four young rabbits treated intracisternally with 2.5 μmole Al maltolate and sacrificed after 72 hours:

Rabbit 1: No neurofibrillary tangles.

Rabbit 2: No neurofibrillary tangles.

Since it was apparent that the young treated rabbits would not demonstrate obvious neurofibrillary pathology at 72 hours, based on this study as well as on our previous observations in numerous other animals [1,3], we limited our examination to just 2 animals in this age group.

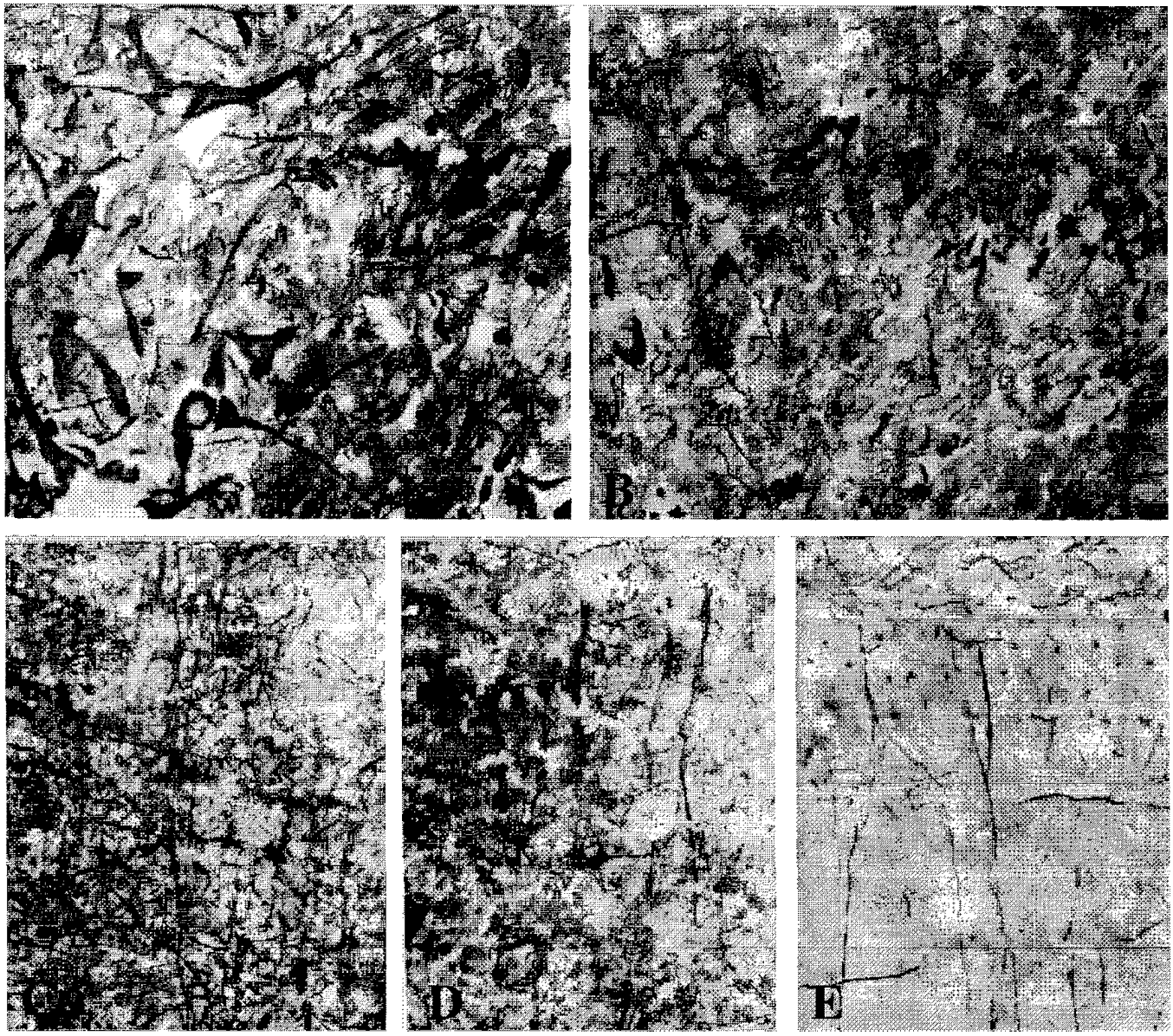
In addition, we evaluated specimens using Bielschowsky's silver-stained sections from the following rabbits treated as indicated. When 2 injections were carried out, they were always 1 hour apart. The final time was the time before sacrifice from the final injection. All rabbits were 4-5 years of age and classified as aged.

Cyclosporin A – Al maltolate;	1 hour -----	3 rabbits
Cyclosporin A – Al maltolate;	3 hours ----	3 rabbits
Cyclosporin A – saline;	3 hours ----	3 rabbits
Saline – Al maltolate;	1 hour ----	2 rabbits
Saline – Al maltolate;	3 hours ----	4 rabbits

Summary: Evidence for neurofibrillary pathology was minimal in all of the rabbits listed above, other than 2 young animals which were sacrificed 5 days following treatment. This agrees with our previous studies where we had observed marked silver positivity in both young and aged animals administered a 2.5 μmole dose of Al maltolate and sacrificed when severe neurological symptoms developed at about 5-7 days [1]. In the young animals the region most affected was the brainstem and spinal cord [2]. In the present group of rabbits, sacrificed at 72 hours, there were early pathological changes in cortical regions in the aged group, but no abnormalities were noted in the younger group. The following photomicrographs (Figure 1) illustrate these findings.

Although, as stated above, there was no evidence of neurofibrillary changes in these animals, there were distinct biochemical changes observed, supporting our hypothesis that opening of the mitochondrial permeability transition pore is an important change in aging. Appendix 1 describes these studies in detail.

FIGURE 1



Fourteen micrometer-thick frozen sections, stained with the Bielschowsky's silver method, a technique for demonstration of neurofibrils. This staining is particularly useful in detecting the so-called "tangles," which are aggregates of neurofilaments that appear in a variety of neuropathological conditions, including Alzheimer's Disease.

All micrographs are printed at the same magnification (300X) for direct comparison; no counterstaining was performed on the sections. **A** and **B** depict the medulla and temporal cortex, respectively, of young rabbits injected intracisternally with 2.5 μmole of aluminum maltolate and sacrificed after 120-144 hours of exposure, by which time they had developed severe symptoms which included hindlimb paralysis and head-tilt. In **A**, distinct densely-stained tangles create bizarre profiles in the medullary nerve cell bodies and processes. The tangles are present in the pyramidal layer of the cortex as well (**B**), but do not appear as dense as those in the medulla.

Panels **C**, **D**, and **E** are brain sections from an aged rabbit, exposed to 2.5 μmole of aluminum for 72 hours. Sections prepared from old animals after this 3-day exposure lack clearly-recognizable tangles in the cortex (**C**) or hippocampus (**E**), although scattered cortical dendrites (examples shown in **D**) display both increased density and thickening. Young animals treated in a similar manner were negative for these changes.

Task 2: Establishment of techniques.

1. Rabbit studies and tissue processing for immunoblot analysis and immunohistochemistry:

Methods for the animal system using the intracisternal route of injection and procurement of tissue with perfusion have been well described in several of our publications [1;3-6]. We have optimized these procedures for the present investigation in order to maximize the number of studies that can be used with the valuable tissue obtained from aged and young rabbits. We found early in the course of our studies that immunoblot analysis on tissue that had been subjected to mild homogenization, followed by ultracentrifugation to obtain mitochondrial and cytoplasmic fractions, was providing us with the most useful information. This tissue was obtained from fresh brain using a ~ 7 mm whole coronal slab containing the hippocampus. The slab was bisected. One side was used for biochemical analysis (following ultracentrifugation), and the other side was frozen rapidly in liquid nitrogen for immunohistochemistry. Details of these methods are given in the attached paper (Appendix 1) on page 4, Materials and Methods, "Animal treatment and tissue processing". Also, conventional electron microscopy was performed on selected mitochondrial and cytosolic isolates in order to confirm and monitor the enrichment of the fractions.

Western blot analysis procedures on mitochondrial and cytosolic fractions have been established, and the details of the methods are given in the attached paper (Appendix 1) on pages 4 and 5 under the heading "Western blot analysis".

2. Caspase-3 enzymatic assay:

This assay is used to ascertain whether the pathway of apoptosis involves caspase-3 activation. The assay contains a caspase-3 fluorogenic substrate, Ac-DEVD-AMC. Active caspase-3 is known to bind to the fluorogenic Ac-DEVD-AMC substrate and to cleave it, releasing the fluorescent AMC. AMC fluorescence is quantified by UV spectrofluorometry, using an excitation wavelength of 380 nm and an emission wavelength range of 420-460 nm.
Caspase-3 activity is inhibited by Ac-DEVD-CHO, which binds strongly to the caspase-3 active site and blocks substrate binding. Hence, Ac-DEVD-AMC is not cleaved and no fluorescence is emitted.

The procedure is as follows:

1) AMC fluorescence: Into a test tube, place 10 μL of Ac-DEVD-AMC, 1 ml of HEPES, 200 μg of cytosolic protein homogenate. Incubate in 37°C for 1 hour, transfer the solution to cuvettes and measure the fluorescence in a spectrofluorometer.

2) Inhibition of the AMC: Use the same procedure as for AMC fluorescence, adding 10 μL of Ac-DEVD-CHO to inhibit the caspase-3.

3) Controls: Deionized water is used as a blank to set the zero reading for the spectrofluorometer and a reading for HEPES is subtracted from the value of the samples. The same amount of the sample is applied as a negative control for AMC reading, without adding 10 μL of Ac-DEVD-AMC.

3. Immunohistochemical methods:

a. Immunohistochemical techniques for Cytochrome C, Bcl-2 and Bax have been developed for use on 14 μm thick frozen sections:

Details for the Cytochrome C immunostaining are given in the attached paper (Appendix 1) on page 5 under the heading "Immunohistochemistry". Details for the Bcl-2 and Bax immunostaining have been published previously [1] (Appendix 2).

b. Immunocytochemical detection of oxidative damage to neuronal DNA:

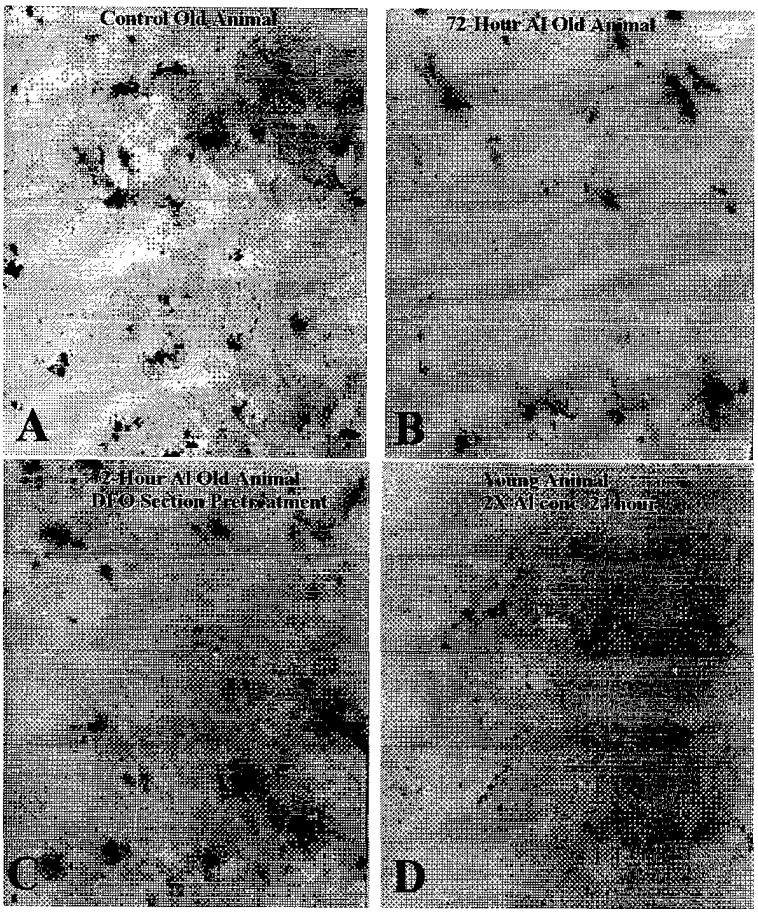
Assessment of oxidative stress by measurement of 8-hydroxy-2'-deoxyguanosine (8-OH dG) was identified as a task for the first year. Our proposed technique was to use gas chromatograph/mass spectrometry. However, the primary standard for this technique, although available commercially, has been back-ordered and has greatly delayed our progress on this aspect. In the meantime we have concentrated our efforts on the crucial study on Cytochrome C in mitochondria in neurodegeneration. We have initiated work on the immunohistochemical detection of 8-OH dG which occurs as a product of oxidative injury to DNA by reactive oxygen species. This assay was performed using a commercially available monoclonal antibody (Trevigen, Gaithersburg, MD). Brain frozen sections (14 μm) were first fixed in cold acetone and washed in phosphate buffered saline (PBS). Sections were then incubated overnight at 4°C in anti-8OH dG antibody diluted in Tris-HCl with 10% normal goat serum. After this primary antibody incubation, sections were washed in PBS and incubated for 1 hour with biotinylated goat anti-mouse antibody at room temperature (Vector Laboratories, Burlingame, CA), washed again in PBS, and then incubated in streptavidin- horseradish peroxidase solution. Sections were finally processed with diaminobenzidine. Our initial results are equivocal and so far we have no definitive evidence of the accumulation of 8-OH dG following Al-induced injury. These studies and the use of gas chromatography/mass spectrometry will be continued over the next few weeks.

c. Evaluation of the redox-active iron histologic procedure:

Fourteen micrometer-thick frozen sections were reacted with diaminobenzidine and hydrogen peroxide, a procedure which according to the literature will reveal redox iron [7]. Sections were left unstained for clear delineation of the reaction product, which appeared in greatest concentration in the caudate nucleus; equivalent views of this region, all at the same magnification of 200 X, are shown in all four panels (Figure 2). **A** = old control rabbit; **B**= old animal exposed for 72 hr to a single injection of Al maltolate (2.5 μmole) via intracisternal injection; **C**= serial section of animal shown in B, but section pretreated for 45 min with desferrioxamine to remove any redox iron; **D**= young animal exposed to twice the dose of Al (5 μmole) for 24 hours.

There is a marginal increase in staining intensity in an Al-treated old animal (panel **B**) compared to a young rabbit treated with twice the Al dose (panel **D**). An untreated control animal demonstrates equivalent staining (panel **A**) to the Al-treated old rabbit (panel **B**) . Desferrioxamine pretreatment of tissue from this old animal does not prevent the staining from occurring (panel **C**). In addition, very little reaction is apparent in the young animal (**D**), despite an exposure to a two times higher Al concentration. We conclude that these results cast doubt on this staining method, since it appears to reveal only endogenous peroxidase activity, such as would be expressed in certain categories of lysosomes. These reactive bodies would be expected to be more numerous, per se, in the neuronal cell bodies of old animals, and far less populous in neurons of young animals, which would be consistent with the staining patterns we have found.

FIGURE 2



Task 3: Cyclosporin A treatment is begun.

The initial proposal was to use intraperitoneal injections of Cyclosporin A prior to the administration of Al maltolate via the intracisternal route. We anticipated some problems with this protocol in that Cyclosporin A must first cross the blood-brain barrier in order to gain exposure to the brain parenchyma. Consequently we decided we would bypass this barrier and administer Cyclosporin A via the intracisternal route. We were pleased to find that there were no adverse clinical events, at least in the short term. Experiments describing the optimization of this Cyclosporin A treatment are given in the attached paper (Appendix 1), page 4, under the heading "Cyclosporin A distribution in brain"; on page 5, under the headings "Results, Cyclosporin A distribution following intracisternal administration into rabbit brain"; and in Table 1 of this paper (Appendix 1).

Summary of the animal experiments:

In addition to completing the tasks that we proposed in the first 12 months, we have also progressed with treating several young and aged animals as suggested for the later studies. A summary of animals treated and sacrificed is as follows in Table 3:

TABLE 3: SUMMARY OF ANIMALS TREATED

Al-maltolate: 100 µl of 25 mM saline
Cyclosporin A: 250 µg in 100 µl saline

Control	Sal/Al 1h	Cs/Al 1h	Sal/Al 3h	Cs/Al 3h	Sal/Al 24h	Cs/Al 24h	Sal/Al 72h	Cs/Al 72h	Cs/Sal 3h
Aged rabbits									
4 rabbits	4 rabbits	4 rabbits	7 rabbits	5 rabbits	7 rabbits	4 rabbits	4 rabbits	1 rabbit	4 rabbits
Young rabbits									
4 rabbits	4 rabbits	4 rabbits	7 rabbits	3 rabbits	1 rabbit	1 rabbit	2 rabbits		

Key: Sal - intracisternal injection of 100 µL saline
Al - intracisternal injection of 100 µL Al maltolate in saline.
Cs - intracisternal injection of 100 µL Cyclosporin A in saline.
The time in hours (h) is the time following the second injection until animal termination.

Results of biochemical studies:

Results of the biochemical studies on most of the animals listed in Table 3 are given below in Tables 4 and 5. Western blot analyses were performed on mitochondrial and cytoplasmic specimens from the hippocampus and frontal cortex. Details of tissue processing and procedures are given in Appendix 1.

TABLE 4: RESULTS OF WESTERN BLOT ANALYSIS FOR AGED RABBITS

	Cytochrome C		*Bcl-2*		*Bax*	
	mitochondria	cytoplasm	mitochondria	cytoplasm	mitochondria	Cytoplasm
Controls	+++	+	+	-	+++	+++
Saline/Al 1h	+++	+	+	-	+++	+++
Cyclo/Al 1h	+++	+	+	-	+++	+++
Saline/Al 3h	++	+++	+	-	+++	+++
Cyclo/Al 3h	+++	+	+	-	+++	+++
Saline/Al 24h	+++	+	+	-	+++	+++
Cyclo/Al 24 h	+++	+	+	-	+++	+++
Saline/Al 72h	+++	+	+	-	+++	+++
Cyclo/Al 72h	+++	+	+	-	+++	+++

Key: + = faint but distinctively positive band
++ = moderately positive band
+++ = intense band

Note: Quantitation of all immunoblots by densitometry is in progress.

TABLE 5: RESULTS OF WESTERN BLOT ANALYSIS FOR YOUNG RABBITS

	Cytochrome C		*Bcl-2*		*Bax*	
	mitochondria	cytoplasm	mitochondria	cytoplasm	mitochondria	cytoplasm
Controls	+++	+	+	-	+++	+++
Saline/Al 1h	+++	+	+	-	+++	+++
Cyclo/Al 1h	+++	+	+	-	+++	+++
Saline/Al 3h	+++	+	+	-	+++	+++
Cyclo/Al 3h	+++	+	+	-	+++	+++
Saline/Al 24h	+++	+	+	-	+++	+++
Cyclo/Al 24 h	+++	+	+	-	+++	+++
Saline/Al 72h	+++	+	+	-	+++	+++
Cyclo/Al 72h	+++	+	+	-	+++	+++

Key: + = faint but distinctively positive band
++ = moderately positive band
+++ = intense band

Note: Quantitation of all immunoblots by densitometry is in progress.

KEY RESEARCH ACCOMPLISHMENTS:

- Cyclosporin A can be administered via the intracisternal route, thus bypassing the blood-brain-barrier and facilitating an understanding of the process of how the the mitochondrial permeability

transition pore is blocked. No adverse effects on the animals have been observed by this treatment.

- Opening of the mitochondrial permeability transition pore, with Cytochrome C release, is an early response to neuronal injury in aged rabbits. This mitochondrial injury probably leads to apoptosis. Injury is caused by the intracisternal administration of Al into these animals; young rabbits are not vulnerable. These findings support the hypothesis that forms the basis of this project.
- As evaluated by immunoblot analysis, the anti-apoptotic protein Bcl-2 is not affected, at time points of 1, 3, 24, and 72 hours following Al maltolate (2.5 μmole) treatment, in either the young or aged rabbits. Bax, which is pro-apoptotic, is also not affected.

REPORTABLE OUTCOMES:

- The attached manuscript (Appendix 1) entitled "Opening of the mitochondrial permeability transition pore with Cytochrome C release is an early response to neuronal injury in aged rabbits", has been submitted for publication to the American Journal of Pathology.
- The following poster was presented on July 11, 2000 at the World Alzheimer Congress 2000, Pivotal Research, in Washington D.C.: Ghribi O, DeWitt D, Forbes MS, Herman MM and Savory J: Cyclosporin A prevents Cytochrome C release in Al-treated aged rabbit brain. The abstract is published in Neurobiology of Aging 2000, 21: S154. This abstract is attached as Appendix 3.
- The following abstract has been accepted for presentation at the 30th Annual Meeting of the Society for Neuroscience to be held in New Orleans, LA, November 4-9, 2000: Ghribi O, DeWitt D, Forbes MS, Herman MM and Savory J: Expression of proteins regulating apoptosis, Cytochrome C, Bcl-2 and Bax in aluminum maltolate-treated rabbit.
- A proposal was submitted to the Jeffress Trust and was funded from July 1, 2000 through June 30, 2002 at a level of $25,000. The Principal Investigator is David DeWitt Ph.D., and John Savory Ph.D. is the Co-investigator. The title of this project is "Aluminum-induced mitochondrial injury, oxidative stress and apoptosis *in vitro*".
- A proposal was submitted to the Alzheimer's Association and is pending. The Principal Investigator is John Savory Ph.D. and the title is "Mitochondrial protection: a preventive and therapeutic approach to neurodegeneration"; $ 239,931, July, 2000 to June, 2003.

CONCLUSIONS:

This initial phase of the project has provided results supporting the original hypothesis, namely that the mitochondrial permeability transition pore is involved in Al-induced neuronal injury and that aged animals are more vulnerable to this injury than are young adults. Our early work has relied more on cell fractionation and western blotting than on immunohistochemistry, although frozen sections have been obtained and stored on all animals used to date. It appears that emphasis on caspase activation will be important in future work, and preliminary studies have been carried out. In a paper published early in 2000 [8] the neuronal endoplasmic reticulum was implicated in apoptosis via the caspase-12 pathway. We are now isolating a cell fraction containing endoplasmic reticulum, and future work should evaluate its role in neurodegeneration. At this stage we see little difference in Bcl-2 and Bax responses to apoptotic stimuli between young and aged rabbits using cell fractions and western blotting approaches. We plan to increase the dose of Al maltolate to see if this observation changes. A very preliminary study suggests that this indeed is the case. Our data also suggest, as is widely accepted, that intracellular calcium control is a key element in triggering apoptosis. We propose that controlling the release of calcium associated with the endoplasmic reticulum will be an important addition to our investigation and we plan to pursue this with collaborative studies using confocal microscopy.

Overall, the research is progressing well; we have most of our techniques established and a goodly supply of frozen tissue available for many additional studies on oxidative stress, caspase activation and apoptosis in the brain.

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APPENDIX 1

Opening of the mitochondrial permeability transition pore with Cytochrome C release is an early response to neuronal injury in aged rabbits

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Running head: opening of MPTP in aged rabbit.

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ABSTRACT

Neurodegenerative diseases such as Alzheimer's disease (AD) are characterized by a progressive and selective loss of neurons, and there is a growing body of evidence which supports a central role of mitochondria in the process leading to apoptotic cell death. Release of cytochrome c from the mitochondria to the cytosol is considered a critical step in apoptosis. Intracerebral administration of aluminum (Al) compounds to rabbits has been shown to induce oxidative stress and apoptosis, particularly in aged animals, but the mechanism is unclear. Here we report that intracisternal injection of Al maltolate induces cytochrome c translocation from brain mitochondria to cytosol as early as 3 hr in aged rabbits, but not in young animals. Pretreatment with cyclosporin A, an inhibitor of the mitochondria permeability transition pore (MTP), blocks cytochrome c release. It would therefore appear that Al-maltolate-induced cytochrome c release results from opening of the MTP. This effect implicates aging as a prerequisite factor, since the MTPs do not open in young animals. Mitochondrial injury may thus represent a primary initiator of neurodegeneration. This is the first in vivo report that aging increases susceptibility to stress-induced opening of the MTP.

Keywords: mitochondria permeability transition pore, cytochrome C, aging, rabbits, aluminum

INTRODUCTION

Alzheimer's disease, the most common human neurodegenerative disease, is characterized by neurofibrillary tangles, neuritic plaque deposition, and selective neuronal death. More recently it has been found that in Alzheimer's disease the brain is under increased oxidative stress; this conclusion is supported both by the observation of increased lipid peroxidation [1;2], protein and DNA oxidation [1] and the observation of iron and aluminum (Al) deposition in the brain of patients with Alzheimer's disease [3]. Evidence of apoptosis also has been seen as an early event preceding the formation of neurofibrillary tangles in Alzheimer's disease [4]. Previous work from our laboratory has demonstrated that the intracisternal injection of Al maltolate in aged rabbits produces neurofilamentous aggregates having many biochemical features in common with neurofibrillary tangles. In addition, Al maltolate injection induces oxidative stress damage and apoptosis, effects which also resemble the changes seen in Alzheimer's disease [5;6].

Recent studies have shown that in Alzheimer's disease, mitochondrial alterations represent a primary event in apoptotic cell death (for review see [7]). Growing evidence furthermore suggests that cytochrome C, a water-soluble peripheral membrane protein of the mitochondria, is released to the cytoplasm following cytotoxic stimuli [8]. The apoptotic process is then activated by the apopotogenic initiators known as caspases; this involves the formation in the cytosol of the complex cytochrome C-Apaf-1 cofactor. The release of cytochrome C into the cytoplasm is reported to be a consequence of an increased permeability of the mitochondria in response to cytotoxic insult [9]. Cytosolic redistribution of cytochrome C is induced by the tumor necrosis factor alpha (TNF α) in primary rat hepatocytes [10]. Cold-injury induced brain trauma [11], transient focal cerebral ischaemia[11,12] and the parkinsonian neurotoxin MPP+ [13] also appear to open the MTP and to release cytochrome C. Cyclosporin A, an immunosuppressant drug, irreversibly inhibits MTP opening [14] and has been shown to block cytochrome c release by TNF α [10]. In addition, treatment with cyclosporin A ameliorates brain damage resulting from transient forebrain ischemia[15,16]or traumatic brain injury [17]

Investigations in our laboratory have focused on neuronal injury resulting from intracisternal administration of the electroneutral Al complex, Al maltolate, to New Zealand white rabbits. This animal system is relevant to a study of human disease in that it reflects many of the histologic and biochemical changes associated with Alzheimer's disease [18]. Rabbits are especially useful in studies of human disease, since their proteins show closer homology with primates than with rodents [19]. Most importantly, *in vivo* studies with this animal model system can address unique changes associated with aging, which almost certainly predisposes the individual to neuronal cell injury [5]. In the present study, we investigate early changes in the expression of cytochrome C, testing the hypothesis that Al administration induces oxidative stress, which is linked to mitochondrial cytochrome C translocation. Pretreatment with cyclosporin A, prevents the opening of the MTP and inhibits translocation of cytochrome C. The effect of aging on the susceptibility of neurons to these events is further examined by comparing changes observed in young adult versus aged animals.

MATERIAL AND METHODS

Animal treatment and tissue processing

Aged (4-5 years old) and young (8-12 months old) female New Zealand white rabbits received intracisternal injections of 100 µL saline, 100 µL Al maltolate and/or 250 µg of cyclosporin A (Sandimmune, Novartis Pharmaceuticals Corporation, East Hanover, NJ) in 100 µL of saline. The injection was carried out under ketamine anesthesia according to the method previously described [6]. The amount of solution injected was the same for the aged and young animals, since the brain weights are similar for both age groups (within 8 -10 g) despite a marked difference in body weight, which ranged from 4-7 kg in the aged animals compared to 2.5 - 4 kg for young rabbits. Each group of rabbits consisted of 4 aged and 4 young adults. Group 1 received saline (0 hr) followed by Al maltolate at 1 hr, and were sacrificed at 2 hrs. Group 2: cyclosporin A (0 hr), Al maltolate at 1 hr, sacrificed at 2 hrs. Group 3: saline (0 hr), Al maltolate at 1 hr, sacrificed at 4 hours. Group 4: cyclosporin A (0 hr), Al maltolate at 1 hr, sacrificed at 4 hrs. Group 5: cyclosporin A (0 hr), saline at 1 hr, sacrificed at 4 hrs. Animals were euthanized and perfused with Dulbecco's phosphate buffered saline (GIBCO, Grand Island, NY) as described previously [20;21]. Brains were removed within 5 minutes after sacrifice and a ~ 5 mm coronal section was cut and bisected to yield two symmetrical hippocampal segments, one for immunohistochemistry and the other for immunoblot analysis. The respective sides chosen for these studies were alternated between successive animals. The brain hemisphere for histology was immediately frozen on a liquid nitrogen-cooled surface and stored in zipper-closure plastic bags at -80° C before sectioning. For immunoblot analysis, tissue from hippocampus and cortex was rapidly dissected, homogenized and subjected to ultracentrifugation as described below.

Cyclosporin A distribution in brain

Cyclosporin A possesses a limited ability to penetrate an undamaged blood brain barrier [22]. The low permeability of Cyclosporin A into the brain is the result of active efflux, from brain capillary endothelial cells, of P-glycoprotein present in their luminal surfaces [23]. By achieving relatively high blood concentrations, it has been shown that it is possible to reach pharmacological concentrations in rat brain [24]. Due to the low penetration of this compound, it seemed desirable to bypass the blood brain barrier by direct administration of cyclosporin A into the cerebrospinal fluid. Thus, we performed a series of experiments in which we injected a solution of Cyclosporin A intracisternally into young (8 - 12 months old) New Zealand White rabbits. Initially we injected 25 µg into one rabbit and observed no adverse effects up to 24 hours, and followed this by injecting 250 µg of Cyclosporin A into the same animal, also via the intracisternal route. There were no observed side effects up to 4 weeks. We then proceeded to evaluate blood and tissue concentrations in other rabbits following the intracisternal administration of 250 µg of cyclosporin A. We evaluated these concentrations at 0.75 hr (n=1), 3 hr (n=1), 6 hr (n=1) and 24 hr (n=1) following the injection. The analysis of tissue homogenates, blood and CSF was carried out using the fluorescence polarization immunoassay on the TdxFLx® clinical analyzer (Abbott Laboratories, Abbott Park, IL).

Western blot analysis

Proteins from both the mitochondrial and cytosolic fractions were extracted as described previously

[8]. Approximately 100 mg of brain tissue from hippocampus were gently homogenized, using a teflon homogenizer (Wheaton), in 7 volumes of cold suspension buffer (20 mM HEPES-KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin and 12.5 μg/ml of N-acetyl-Leu-Leu-Norleu-Al). The homogenates were first centrifuged at 750 g at 4 C° for 5 min, and then at 8000 g for 20 min at 4 C°. The 8000 g pellets were resuspended in cold buffer without sucrose and used as the mitochondrial fraction. The supernatant was further centrifuged at 100,000 g for 60 min at 4 C° and used as the cytosolic fraction. Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, Illinois, USA). The proteins (7.5 μg) from both the mitochondrial and cytosolic fractions were separated by SDS-PAGE (15% gel) under reducing conditions followed by transfer to polyvinylidene difluoride membrane (Millipore), at 300 mA for 210 min in transfer buffer (20 mM Tris-base, 150 mM glycine, 20% methanol). Following transfer, the membranes were incubated with mouse monoclonal antibody to human cytochrome C (Pharmingen, San Diego, California) at a 1:250 dilution. Following washes with TBS containing 0.1% Triton X-100, the blots were developed using enhanced chemiluminescence (Immun-Star goat anti-mouse IgG detection kit, Bio-Rad, Hercules, CA).

Immunohistochemistry

Serial 14 μm-thick coronal frozen sections were cut at the level of the hippocampus and stored at -80° C prior to immunostaining. The sections were air-dried at room temperature, fixed in cold acetone for 10 min, treated with 1% hydrogen peroxide in phosphate buffer saline (PBS) and incubated with blocking solution of 1.5% normal serum in PBS. Subsequently, sections were reacted overnight at 4°C with mouse monoclonal antibody against cytochrome C (7H8.2C12, Pharmingen, San Diego, CA) at a 1:500 dilution. After washing with 50 mM Tris buffered saline (TBS) and incubation with the biotinylated secondary antibody, sections were processed with a Vectastain Elite avidin-biotin complex technique kit (Vector Laboratories, Burlingame, CA) and visualized by 3,3′-diaminobenzidine/hydrogen peroxide, with light hematoxylin counterstaining, all procedures were performed at room temperature unless otherwise noted. For negative controls, sections were incubated in the absence of primary antibodies.

RESULTS

Cyclosporin A distribution following intracisternal administration into rabbit brain

Concentrations of Cyclosporin A following intracisternal injections of 250 μg in 100 μL of solution are given in Table I, and provide evidence that we were able to achieve significant concentrations of Cyclosporin A in brain tissue by the intracisternal route of administration, with no apparent side effects.

Western blot analysis

The immunoreactivity of cytochrome C was evident as a single band of 15 KD molecular weight, thus confirming the specificity of the monoclonal antibody for cytochrome C used in the present study. In aged animals, cytochrome C immunoreactivity was only faintly detectable in the cytosol at 1 hr after Al treatment (Figure 1A, lane 1) and was strongly reactive in the mitochondria (Figure

1A, lane 2). At 3 hrs, there was a significant cytosolic increase in cytochrome C immunoreactivity (Figure 1A, lane 3) and a corresponding decrease in the amount of cytochrome C in the mitochondria (Figure 1A, lane 4). Pretreatment with cyclosporin A, 1 hr prior to Al administration, prevented the translocation of cytochrome C in the cytoplasm (Figure 1A, lane 5) with a corresponding increase in mitochondrial cytochrome C (Figure 1A, lane 6). In young animals, cytochrome C was barely detectable in the cytoplasmic fraction at 1 hr (Figure 1B, lane 1) but was intensely present in the mitochondrial fraction (Figure 1B, lane 2), and was only slightly increased in the cytosol at 3 hrs following Al injection (Figure 1B, lane 3). Cyclosporin A administration had no effect on this slight increase of cytochrome C in the cytoplasm (Figure 1B, lane 5).

Cytochrome C immunoreactivity

We examined the immunohistochemical localization of cytochrome C in the pyramidal cell layer of the hippocampus (Figure 2A) of all animals. No reaction for cytochrome C was observed in sections processed without incubation with primary antibody (data not shown). There was distinct punctate cytosolic immunostaining in the examined area in the saline-Al treated animals (Figure 2B). However, light microscopic visualization did not enable us to distinguish the cytoplasmic from the mitochondrial localization of cytochrome C in the cyclosporin and/or Al-treated rabbits.

DISCUSSION:

The current study provides the first *in vivo* evidence that aging increases susceptibility to stress-induced opening of the MTP, as assessed by cytochrome c release from mitochondria into the cytosol in neurons of aged rabbits treated intracisternally with Al-maltolate. The observations made in this study were carried out on brain tissue homogenates by immunoblots of isolated mitochondrial and cytosolic fractions. Confirmation was made by immunohistochemistry, using the same monoclonal antibody to cytochrome C. The appearance of cytochrome c in the cytosol of aged rabbit brain occured within 3 hours following the Al-maltolate treatment, and was dramatically reduced by pretreatment with cyclosporin A, an agent known to block opening of the MTP[14]. Release of cytochrome C into the cytosol is reported to result from its diffusion following cytotoxic stimuli from its location through the MTP between the inner and outer mitochondrial membranes [25,26]. The MTP is a large proteinaceous channel which forms between the inner and the outer membranes of mitochondria and can nonselectively pass solutes up to 15 KDa [27,28]. Opening of the MTP rapidly causes depolarization, uncoupling of oxidative phosphorylation, and subsequent pronounced mitochondrial swelling[29,30]. Numerous agents, including calcium ions, inorganic phosphate and oxidant chemicals are known to promote MTP opening[29,30]. Although the exact composition of the MTP is not yet determined, it involves proteins from the cytosol, the outer and the inner membranes and the matrix (cyclophilin D)[30,31,32] Cyclosporin A is reported to to block the MTP opening by specifically binding mitochondrial cyclophilin [30,33].

In the present study, inhibition by cyclosporin A of the translocation of cytochrome C into the cytosol following Al-maltolate treatment suggests that the observed cytosolic cytochrome C originates from mitochondrial release, as a consequence of opening of the MTP. In addition, ultracentrifugation of the cytosolic fractions at 100 000 g for 60 min makes it unlikely that the

observed cytosolic presence of cytochrome C following Al-maltolate perfusion is derived from other sources. Indeed, organelles such as endoplasmic reticulum and ribosomes constitute the main source of cytochrome C synthesis. However, most of these organelles have been removed from the cytosolic samples by the ultracentrifugation procedure used in the present experiments. Nevertheless, we could not exclude the possibility that mechanisms other than the opening of the MTP might release cytochrome C, and further studies will be required.
In contrast to the response in aged rabbits, young animals exhibited only a slight cytosolic immunoreactivity of cytochrome c following Al administration, as analyzed by western blot analysis. Treatment with cyclosporin A did not affect the distribution of cytochrome C .

The mechanism of the Al effect is unclear at this time, but could be related to increased production of reactive oxygen species that are known to open the MTP. Previously we have demonstrated the presence of increased oxidative stress in this animal model [5].
Mounting evidence implicates mitochondria as a critical site at which different apoptotic signals converge. Mitochondria are assumed to be involved in apoptosis by opening the MTP, thus releasing cytochrome c to the cytoplasm where it activates caspase 3, which has been shown in turn to trigger apoptosis [8]. In addition, impairment of electron transport leads to a decrease in ATP production and an increase in free radical or reactive oxygen species production, conditions which are potentially lethal to cells [7]. Furthermore, opening of the MTP leads to a release of Ca^{2+}, and the probability of pore opening is increased as intramitochondrial Ca^{2+} increases [34,35] .
In the present study, although cytochrome C release in aged rabbits is suggested to result from the opening of the MTP, as assessed by the ability of cyclosporin A to inhibit the release, the mechanism by which Al-maltolate administration induces the opening of the MTP remains to be determined. Al-maltolate-induced cytochrome C release may result from an alteration of the mitochondrial membrane potential, generation of reactive oxygen species, or perturbation of Ca^{2+} hemostasis. Recently, it was reported that cytotoxic insult leads to Bcl-2 overexpression in the endoplasmic reticulum and an increased migration of Ca^{2+} to the mitochondria. The extra Ca^{2+} in the mitochondria is rapidly expelled to the cytosol [36]. One other possible mechanism is that Al-maltolate causes overexpression of the proapoptotic Bax, which then translocates from the cytosol to the mitochondria and leads to cytochrome C release. Indeed, in several models of cell death the overexpression of Bax induces its translocation from the cytosol to the mitochondria [37,38] with a subsequent release of cytochrome C [39]. The observation that in young animals, cytochrome c is not released to the cytosol following Al-maltolate administration, indicates that in this case the mitochondria resist opening of the pores. This difference in MTP pore opening between aged and young animals indicates that aging is a facilitating factor in the opening of MTP. This difference in the opening of MTP and subsequent cytochrome C release may account for the deleterious effects following Al-maltolate administration in aged rabbits that we reported previously [5].

In conclusion, the present findings implicate mitochondrial injury, as assessed by cytochrome C release, as an early event in Al-induced neuronal injury, and in addition implicate the opening of the permeability transition pore as an important component of this process. Since the Al-rabbit model system demonstrates many of the biochemical and neurofibrillary changes seen in Alzheimer's disease and related neurodegenerative disorders, we propose that MTP-related processes of mitochondrial

injury may also be important early events in these human diseases.

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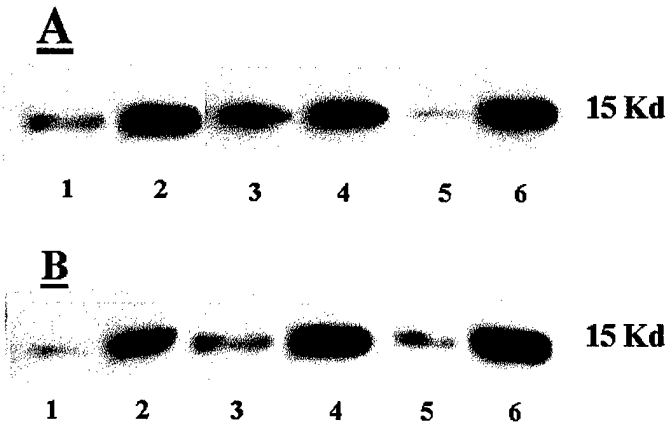
Table I. Time concentrations of Cyclosporin A in hippocampus, blood and CSF of young rabbits at 45 min, 3 hrs, 6 hrs or 24 hrs following the intracisternal injection of 250 μg Cyclosporin A.

Figure 1. Western blot analysis of cytosolic and mitochondrial cytochrome C in hippocampus of aged (A) and young rabbits (B). Cytosolic (Lane 1) and mitochondrial (Lane 2) fractions from rabbits sacrificed 1 hr after Al-maltolate injection, showing no cytochrome C release from mitochondria to the cytosol. Lane 3: cytosolic and Lane 4: mitochondrial immunoreactivity in animals sacrificed 3hr after Al-maltolate injection; cytochrome C is released to the cytosol in old but not in young rabbits. Lane 5 : cytosolic and Lane 6: mitochondrial immunoreactivity in animals pretreated with cyclosporin A and sacrificed 3 hr after Al-maltolate treatment; Cyclosporin A inhibits cytochrome C release from mitochondria to cytosol in old animals.

Figure 2. Cytochrome C immunostaining (with hematoxylin counterstaining) in hippocampus of coronal brain sections of aged rabbits. A: Pyramidal neurons in CA1, the area of hippocampus examined for immunostaining is enclosed in a box. B: Cytochrome C punctate immunoreactivity.as observed by light microscopy in a section from an old rabbit injected with saline -Al maltolate and sacrified 3 hrs later(x 800).

Table I.

Time	*Hippocampus*	*Blood*	*CSF*
45 min	15.1 μmole/kg	0.05 μM	>59.8 μM
3 hours	40.7 μmole/kg	0.04 μM	53.2 μM
6 hours	0.84 μmole/kg	0.018 μM	52.5 μM
24 hours	0.28 μmole/kg	0.018 μM	0.27 μM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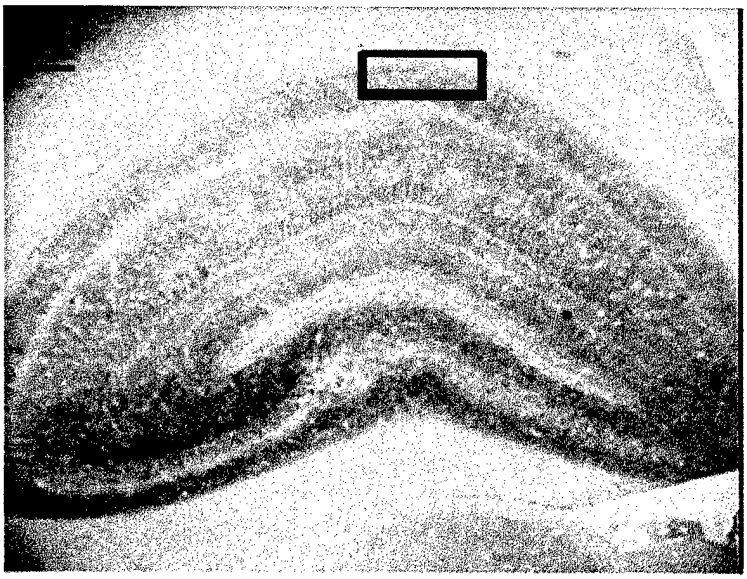

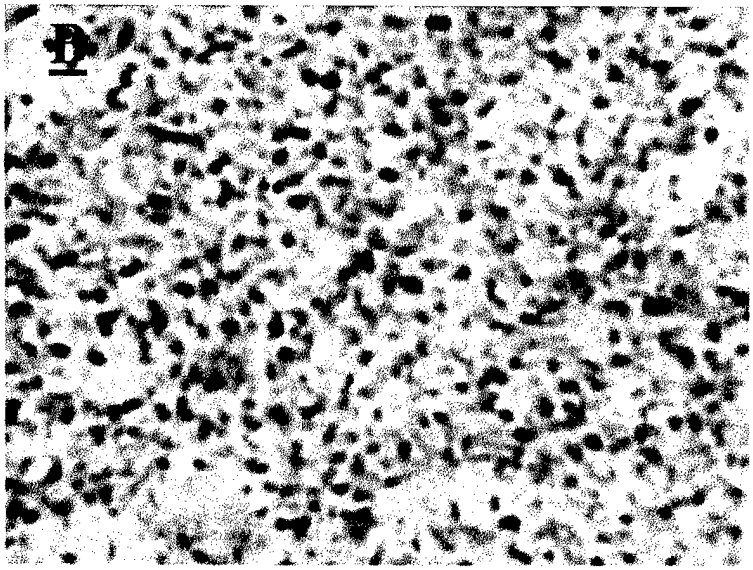
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Appendix II

Age-Related Hippocampal Changes in Bcl-2:Bax Ratio, Oxidative Stress, Redox-Active Iron and Apoptosis Associated with Aluminum-Induced Neurodegeneration: Increased Susceptibility with Aging

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Abstract: *We propose that aging is an important factor in the susceptibiliy of neurons to oxidative stress and to subsequent apoptosis. In the present report we demonstrate that aged rabbits treated intracisternally with aluminum maltolate exhibit intense intraneuronal silver positivity indicative of the formation of neurofilamentous aggregates, together with oxidative stress. These changes occur in the CA1 region of the hippocampus as well as in cerebral cortical areas. Apoptosis, measured by the TUNEL in situ technique, colocalizes with oxidative stress. Young animals treated with aluminum show few of these alterations, while age-matched controls are essentially negative. Further studies on the time course of these and related changes demonstrate that oxidative stress and redox-active iron accumulation in hippocampal neurons occur very rapidly, within a period of 3 hours, and increased in intensity at 72 hours. Changes suggestive of apoptosis are seen by 24 hours and are pronounced at 72 hours. In aged animals there is an initially intense immunopositivity at 3 hours for Bcl-2, with negative staining for Bax. By 72 hours, when apoptosis is strongly evident, Bcl-2 is negative and Bax strongly positive. In contrast to the aged rabbits, young animals treated similarly with aluminum exhibit much less oxidative stress with no apoptosis, and maintain Bcl-2 immunopositivity and negative Bax staining. Our findings strongly support the key role that oxidative damage plays in the process of neurodegeneration and in the increased vulnerability to aluminum-induced injury in the aged animal. These are novel observations which may have important implications for aiding in our understanding of the pathogenesis of neurodegeneration occurring in Alzheimer's disease.*

Key Words: Aluminum, Cerebral Neurodegeneration, Aged Rabbits, Oxidative Stress, Redox-Active Iron, Apoptosis, Bcl-2, Bax

INTRODUCTION

An understanding of the pathogenesis of Alzheimer's disease (AD) continues to elude neuroscientists despite intense research activity, particularly in the well-defined genetic forms of the disease. The characteristic neuropathological features consisting of neurofibrillary tangles and neuritic plaques are important events (for a recent review see Trojanowski *et al.*, 1997), but may represent later markers of a more fundamental early process. Recent studies of brain tissue from AD patients have demonstrated evidence of oxidative damage (Smith *et al.*, 1996) which may be linked to the accumulation of redox-active iron (Smith *et al.*, 1997). Neuronal DNA damage with distinct morphological features of apoptosis also appears to constitute an early event preceeding neurofibrillary tangle formation (Su *et al.*, 1997), and there is evidence of up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-x, with some changes in the pro-apoptotic proteins of the Bcl-2 and caspase families (Kitamura *et al.*, 1998). The time course of such changes and their relationship to neurofibrillary tangle and neuritic plaque formation cannot be determined with the limited information available using a single time

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point, an intrinsic limitation resulting from the use of human autopsy tissue. We report here experiments on rabbits which allow such temporal relationships to be evaluated. Rabbits are particularly relevant to the study of human disease since, as has been reported recently, their proteins have a closer homology with primates than with rodents (Graur *et al.*, 1996) and provide a unique animal system for the consistent production of neurofibrillary pathology (Klatzo *et al.*, 1965, Savory *et al.*, 1996). Moreover rabbits, along with cats, develop intraneuronal neurofilamentous aggregates (NFD) in response to the intracerebral administration of aluminum (Al) salts, whereas rodents do not develop these lesions (Yokel 1989). The rabbit has been the most widely used experimental animal for such studies because of its vulnerability to Al and its availability (Klatzo *et al.*, 1965, Yokel 1989).

For toxicity studies using Al, the selection of an appropriate Al compound is important. We have employed the electroneutral Al maltolate complex (Finnegan *et al.*, 1986) for toxicological studies for a number of years (Bertholf *et al.*, 1987), since this compound can deliver a predicted amount of free aqueous Al^{3+} at a physiological pH (Martin 1991). In contrast, most other Al salts, such as $AlCl_3$, produce insoluble complexes at a neutral pH (Martin 1991). We have shown that the intracisternal administration of an aqueous solution of Al maltolate compound into rabbits will produce NFD, with many biochemical similarities to those seen in AD, in that the argyrophilic lesions contain abnormal tau, hyperphosphorylated neurofilament protein, amyloid precursor protein, A_β, ubiquitin and α_1-antichymotrypsin (Huang *et al.*, 1997; Savory *et al.*, 1995). In this system, hyperphosphorylation of protein constituents of the neurofilamentous aggregates appears to be a secondary process (Savory *et al.*, 1996) and not the primary event as suggested by other investigators (Iqbal *et al.*, 1994, Matsuo *et al.*, 1994). In young adult rabbits, the foci of NFD are not detected in the hippocampus, as is often evident in AD (Huang *et al.*, 1997). However, using aged (4-5 years old) rabbits, we now report that the hippocampus is usually affected following Al administration, as demonstrated by NFD, oxidative stress damage and apoptosis, and that these events are infrequent or not observed in the Al-treated young adults.

Because of these encouraging results, we have investigated the time course for the detection of oxidative damage and redox iron accumulation and their relationship to evidence of apoptosis, with particular attention to changes as early as 3 hours after Al administration. We also have examined how the antiapoptotic Bcl-2 and the proapoptotic Bax proteins respond to Al-induced injury, since an altered response of these two related proteins could constitute a key defect in the aged neuron, leading to increased susceptibility for oxidative damage and apoptosis. Because aging appears to be an important factor in experimental neurodegeneration, we have compared the results in aged rabbits with those in young adults, using an Al/rabbit system which is unique in its ability to reliably produce neurofibrillary pathology in the brain.

MATERIALS AND METHODS

Neuroanatomical Regional Susceptibility to Silver-Positive Al-Induced NFD in Eleven Brain Regions at 5-7 Days after Al

Animal Studies and Tissue Processing. The intracisternal injection of Al-maltolate into New Zealand white rabbits has been described previously (Savory *et al.*, 1995). Four aged (4-5 years old) and three young (8 months old) female rabbits were injected with 20µL of 25 mM Al-maltolate/kg body weight (13.5 µg/kg of elemental Al), and two similarly aged old and two young females were treated with an equivalent volume of saline (total n = 11). Female rabbits were used for consistency in the experimental design and also because the incidence of Alzheimer's disease has been reported to be as much as twice as high in women compared to men (Tomlinson 1992). The youngest rabbits can be considered juveniles, since females of this strain reach breeding age by 6 months (Harkness *et al.*, 1985). All animals were sacrificed on days 5 to 7, by which time the Al-treated group had developed severe neurological symptoms. Paraffin-embedded tissue was employed for these initial studies, with perfusion, tissue processing, reagents and staining methods as utilized in our earlier reports (Katsetos *et al.*, 1990; Savory *et al.*, 1996). Serial 5µm thick sections were cut coronally through each cerebrum from the frontal pole through the occipital pole, as well as axial sections through the brainstem. The anatomical localization of cerebral cortex, hippocampus and midbrain regions on the sections was accomplished using the designation outlined in an Atlas of the rabbit brain and spinal cord (Fig.1) (Shek *et al.*, 1986).

Quantitation of Silver-Positive Neurofilamentous Aggregates. Twenty 5 µm-thick paraffin sections were chosen at similar regular intervals from each brain (n = 11), mounted on glass slides and stained with the Bielschowsky's silver impregnation method for axons adapted for paraffin (Katsetos *et al.*, 1990). From these slides, quantitation of the silver-positive NFD was performed on 11 brain regions from each animal (detailed in the legend of Fig. 2A). Images were captured using an Olympus BH-2 light microscope (Olympus Corp., Lake Success, NY), video camera (Model JE-3662HR, Javelin Electronics, Los Angeles, CA), and a MacIntosh computer with Adobe Photoshop MacIntosh version 2.5.1). A

minimum of 18 fields (a total area of approximately 1 mm^2) was captured and printed at 40x magnification in one tissue section from each of the eleven brain regions. The number of NFD per mm^2 on the computer-generated printed photomicrographs was counted by two independent observers; the inter-observer variability was within 2-5% (Fig. 2A)

Distribution of NFD, PHF-1, SMI-31, Oxidative Stress, Redox-Active Iron and Apoptosis in Four Cerebral Regions at 5-7 Days after Al

Animals for these studies were the same as used for the regional susceptibility section above. Table I outlines the four brain regions analyzed for Bielschowsky's silver-positive NFD as well as for the immuno- or histochemical localization of PHF-1, SMI-31, oxidative stress, redox-active iron and apoptosis. These animals (n = 11) are the same experimental group as those use for NFD quantitation (Fig. 2a). The presence of neurofilament protein and abnormally phosphorylated tau (PHF-1) was evaluated in 5 μm thick paraffin sections with procedures used previously (Savory *et al.*, 1996); a Clonal PAP kit (Sternberger Monoclonals Inc., Baltimore, USA) was employed. The monoclonal antibody (mAb) SMI-31 (Sternberger Monoclonals Inc.) was diluted 1:1000 (IgM concentration 2.7 μg/ml), and mAb PHF-1 (gift from Dr.

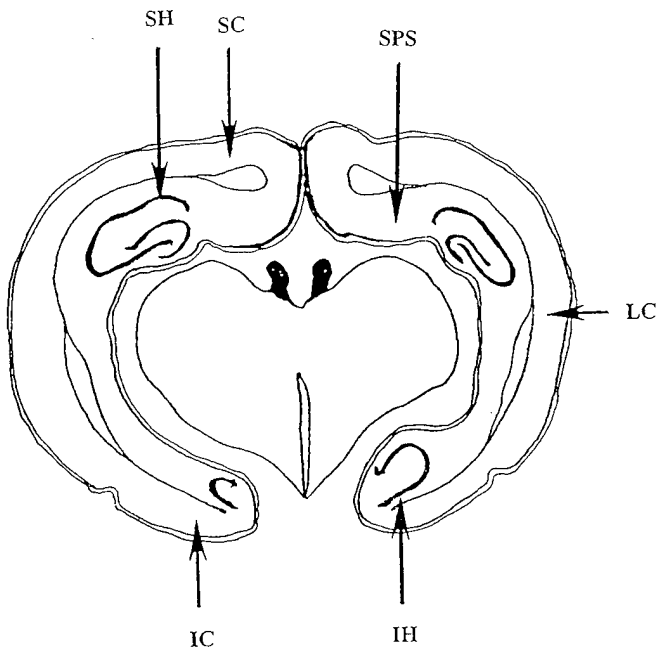


FIG. 1. Schematic representation of some of the rabbit brain regions studied. SC: Superior cortex; LC: Lateral cortex; IC: Inferior cortex; SPS: Stratum pyramidale subiculum; SH: Superior stratum pyramidale hippocampus (CA1 region); IH: Inferior stratum pyramidale hippocampus (CA1 region). Cerebral cortical areas which were analyzed are at the level of SH and IH.

TABLE 1. Distribution of neurofibrillary pathology and oxidative stress products in cerebral cortical and hippocampal brain regions in Al- and saline-treated young and aged rabbits at 5-7 days.

		BRAIN REGIONS			
		Superior Cortex*		Subiculum	
		NFD: PHF-1, SMI-31	Oxidative Stress	NFD: PHF-1, SMI-31	Oxidative Stress
Age	Treatment				
8 mo	Saline (n = 2)	-	-	-	-
8 mo	Al-maltolate (n = 3)	+	-	+	-
4 - 5 yrs	Saline (n = 2)	-	-	-	-
4 - 5 yrs	Al-maltolate (n = 4)	+ + +	+ + +	+ + +	-
		Superior Hippocampus (CA1)		Inferior Hippocampus (CA1)	
		NFD: PHF-1, SMI-31	Oxidative Stress	NFD: PHF-1, SMI-31	Oxidative Stress
8 mo	Saline (n = 2)	-	-	-	-
8 mo	Al-maltolate (n = 3)	-	-	+ +	-
4 - 5 yrs	Saline (n = 2)	-	-	-	-
4 - 5 yrs	Al-maltolate (n = 4)	+	+ + +	+ + +	+ + +

*At level of superior and inferior segments of the hippocampus. See Materials and Methods for details of treatment and techniques used.
- = Absent or rare.
+ = Few.
++ = Moderate.
+++ = Many.
n = Number of rabbits.
Saline = Saline-treated controls.
Data for quantitation of silver-positive NFD and apoptosis are summarized in Fig. 2.

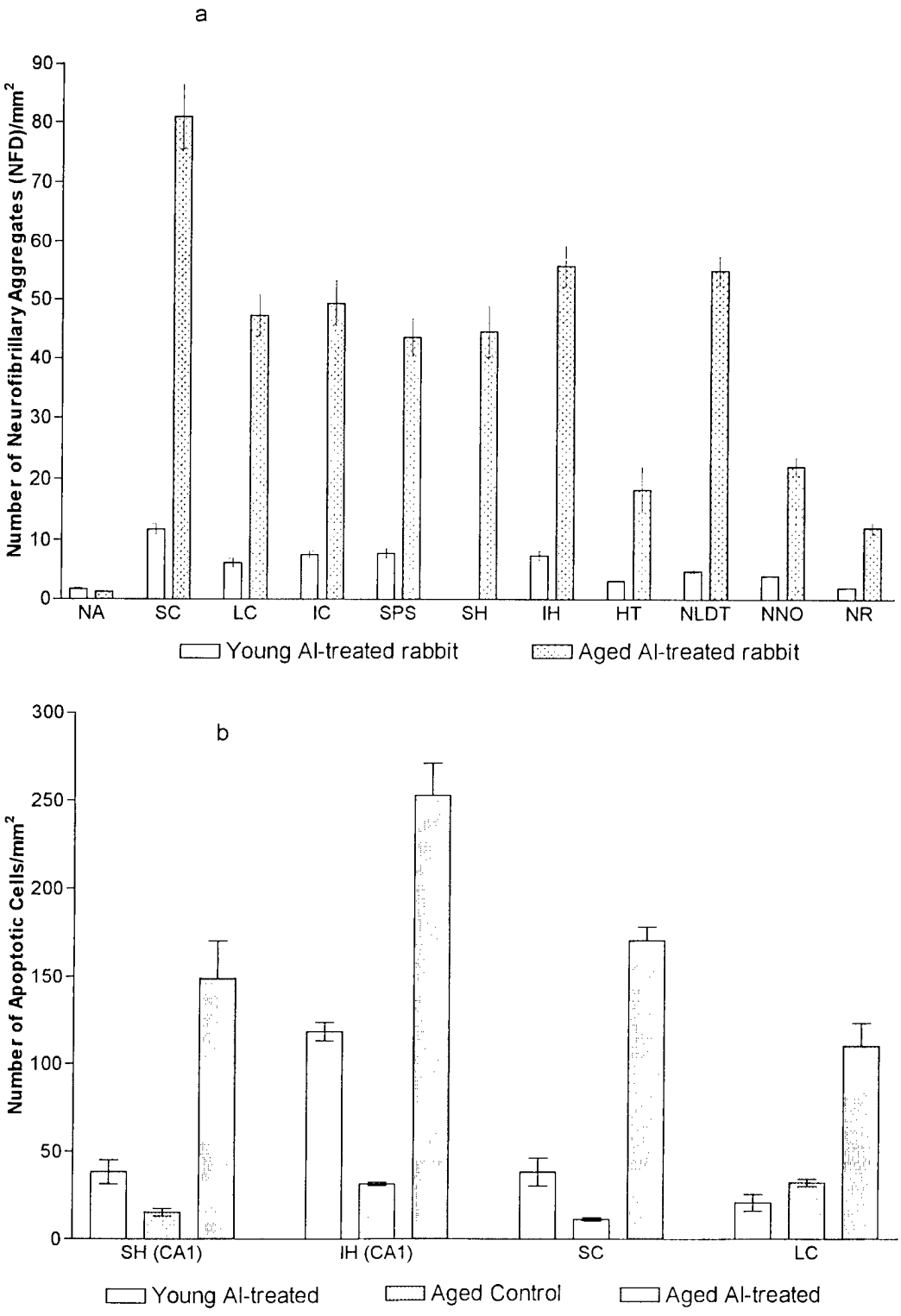


FIG. 2a. Composite graph of the quantitation of silver-positive neurofibrillary aggregates (NFD) in 11 brain regions in aluminum (Al)-treated aged (4-5 years) and young (8 months) rabbits. NA: Nucleus amygdalae; SC: Superior cortex; LC: Lateral cortex; IC: Inferior cortex; SPS: Stratum pyramidale subiculum; SH:Superior stratum pyramidale hippocampus,CA1 region (no NFD were seen in young animals in this region); IH: Inferior stratum pyramidale hippocampus (CA1 region); HT: Hypothalamus; NLDT: Nucleus lateralis dorsalis thalami; NNO: Nucleus nerve occulomotorius; NR: Nucleus ruber. Bars represent SD. Cerebral cortical areas have been analyzed at the level of SH and IH. Bielschowsky's silver impregnation method adapted for paraffin. See text for the statistically-significant findings.**FIG. 2b.** Graph of the quantitation of apoptosis in 4 brain regions of a young (8 months) Al-treated, an aged (4-5 years) Al-treated and a similarly aged saline-treated rabbit. Paraffin-embedded sections have been analyzed. SH: Superior stratum pyramidale hippocampus (CA1); IH: Inferior stratum pyramidale hippocampus (CA1); SC: Superior cortex; LC: Lateral cortex. Cerebral cortical areas were analyzed at the level of SH and IH. (TUNEL reaction, fluorescence). See text for the statistically-significant results.

Peter Davies) was diluted 1:15 (IgG1 concentration 4.6 µg/ml). Sections were dehydrated, lightly counterstained with hematoxylin and mounted.

Oxidative Stress. The presence of oxidative stress was examined in frozen sections by staining free carbonyl groups using the method developed by Smith et al. (Smith *et al.*, 1996). The monoclonal antibody was kindly provided as a gift by Dr. Mark A. Smith (Case Western Reserve, Cleveland, OH). 14 µm thick frozen sections from Al-maltolate treated aged rabbits were mounted and dried on glass slides. They were then hydrated through ethanols to 95%, treated with 3% hydrogen peroxide in methanol for 30 minutes at room temperature, continued through graded ethanols, starting at 70%, to 50 mM Tris buffered saline (TBS) at pH 7.6, treated with 0.01% 2,4-dinitrophenylhydrazine in 2N HCl for 1 hr at room temperature, and rinsed in TBS. Subsequent treatment of the sections was with 10% normal goat serum (NGS) in TBS for 1 hr at room temperature to block non-specific binding sites, then with rat anti-DNP antibody (1:100) overnight at 4^{0}C. Rinsing was performed with 1% NGS in TBS, then incubation with goat anti- rat secondary antibody (1:50) for 30 minutes at room temperature, rinsing with 1% NGS in TBS, treatment with rat-peroxidase anti-peroxidase (PAP) diluted 1:250 for 1 hr at room temperature, and finally, development with 3,3'-diaminobenzidine (DAB)/hydrogen peroxide. The sections were not counterstained.

Quantitation of Apoptosis. Detection of apoptosis by fluorescence was performed on paraffin sections from the 11 animals using the TUNEL technique (Promega apoptosis detection kit, Madison, WI), as modified in our laboratory (Rao *et al.*, 1998). The sections were 5 µm thick, mounted on glass slides and not counterstained. However, adjacent tissue sections were stained with hematoxylin-eosin for comparison. Sections were photographed at 520 nm and 400x magnification onto 35 mm color slides which were scanned using Adobe Photoshop 3.0 (Abode Systems Incorporated, Mountain View, CA) and a Nikon scanner (Nikon LS-3510 AF) and the captured images used for quantitation. Cells were counted as positive for apoptosis based on well-defined criteria as outlined previously (Rao *et al.*, 1998). The number of apoptotic cells were evaluated by two independent observers. Since only 4 brain regions showed evidence of apoptosis, our analysis was restricted to these 4 areas. A minimum of 18 fields were photographed from 4 brain areas at an initial magnification of 400x (see legend, Fig. 2b, for the regions selected, and Fig. 1 for the criteria used for neuroanatomic location). A total area of 1 mm^2 for each brain region was evaluated and the number of apoptotic cells was represented as cells/mm^2. The interobserver variability was 2-5%. The statistical significance ('t' value') was calculated using an SAS package (SAS Institute,Inc., Cary, NC).

Colocalization of NFD and Apoptosis. Similar paraffin-embedded sections from Al-maltolate treated aged rabbits containing hippocampal and cerebral cortical regions were first silver-stained for NFD by the Bielschowsky's silver impregnation method and then stained for apoptosis by the TUNEL method, as above. Slides were examined for colocalization of NFD and apoptosis within the same neuron of the CA1 region.

Colocalization of Abnormal Tau (mAb PHF-1 Positivity) and Apoptosis. Similar paraffin- embedded sections were first stained with the PHF-1 mAb and developed with DAB reagent, followed by staining for apoptosis by the TUNEL method. Sections were examined for colocalization of PHF-1 immunopositivity and apoptosis in the same neuron of the CA1 region.

Statistical Analyses of NFD and Apoptosis. Significant changes in number of silver-stained NFD and cells positive for apoptosis between the aged and young rabbits treated with Al-maltolate were calculated using the SAS package ANOVA and General Linear Models programs (SAS Institute, Inc., Cary, NC) (summarized in Fig. 2A,B), as were statistical probabilities between the results of the 2 observers.

Time-Sequence Studies of Bcl-2:Bax, Oxidative Stress, Redox-Active Iron and Apoptosis in CA1 of the Superior Hippocampus at 3, 12, 24, 48 and 72 Hours after Al

In another experiment, ten aged (4-5 years old) and 10 young (8 months old) female rabbits were injected as described above with 20µL Al-maltolate/kg body weight. Controls were 5 aged (4-5 years) and 5 young (8 months) female rabbits, treated with the same volume of 75 mM maltol (total, n = 30). Both solutions were made up in saline. Using the above animals, two Al-treated and 1 maltol-treated rabbits of the young and old age groups were sacrificed at each time interval of 3, 12, 24, 48, and 72 hours after injection. They were euthanized, then gravity-perfused from a height of one meter with 200 ml of 0.9% saline at 37^{0}C, followed by 250 ml of phosphate buffered saline (pH, 7.4) at 37^{0}C. The brains were immediately frozen in a slurry of 50:50 dry ice:isopentane and stored at -85^{0}C. Frozen tissue sections were used for all subsequent evaluations. Serial 14µm thick sections were cut coronally through each brain from the frontal pole through the occipital pole, and mounted on glass slides. Sections were not counterstained.

The superior segment of CA1 was chosen for study since it is frequently involved with NFD in the aged

animals and is easily identified, even under fluorescent microscopic examination. Detailed examination of CA2 and CA4 was not within the scope of this project. This region was photographed at an initial magnification of 400x following immuno- or histochemical procedures for the localization of Bcl-2 and Bax, oxidative stress reaction products, redox-active iron and apoptosis,.

Bcl-2 and Bax. Frozen sections were air dried at room temperature and stored at 37C overnight. They were treated with 3% hydrogen peroxide (in methanol) for 10 minutes, washed with 50 mM tris-buffered saline (TBS, pH, 7.4) for 20 minutes, and incubated with 10% NGS in TBS for 1 hour at room temperature to block non-specific binding sites. Subsequently they were incubated overnight at 4C with Bcl-2 monoclonal antibody (NCL-Bcl-2, Vector Laboratories, U.S.A.) diluted 1:25, or Bax monoclonal antibody (Mouse Anti-Bax, Zymed, U.S.A.) diluted 1:500. After washing with TBS and incubation with the biotinylated secondary antibody, sections were processed with a Vectastatin avidin-biotin complex technique elite kit (Vector) and visualized by 3,3-diaminobenzidine/hydrogen peroxide.

Oxidative Stress and DNA Fragmentation. Both were detected independently in frozen sections with essentially the same methods as described above.

Redox-Active Iron. Redox-active iron was directly demonstrated by incubation for 45 minutes of the frozen tissue sections with 3% hydrogen peroxide and 0.75 mg/ml 3,3'-diaminobenzidine with the method described by Smith *et al.* (1997). The final staining development involves the same oxidation reaction of 3,3'-diaminobenzidine as in the peroxidase-antiperoxidase/3,3'-diaminobenzidine technique.

Colocalization of Oxidative Stress and Apoptosis. Similar frozen sections were first stained for the presence of oxidative stress as described above and subsequently restained for apoptosis by the TUNEL method.

Table 2 summarizes the animals and findings of these time sequence studies in CA1 of the hippocampus.

RESULTS

Neuroanatomical Regional Susceptibility to Al-Induced Neurodegeneration

Assessment of Neurofibrillary Degeneration (NFD). Widespread argyrophilic NFD was found in a number of brain regions in Al-treated aged and young rabbits (Table1, Fig.2a), although quantitatively the aged animals were affected to a much greater extent (Fig. 2a). No NFD was detected in the saline-treated controls in either the young or aged groups (Table 1). NFD was observed (Fig. 2a) mostly in the superior (SC), lateral (LC), and inferior (IC) cerebral cortices (at the level of the superior and inferior segments of the stratum pyramidale hippocampus); in the stratum pyramidale subiculum (SPS); in the CA1 region of the superior (SH) and inferior (IH) segments of the stratum pyramidale hippocampus; and in the nucleus lateralis dorsalis thalami (NLDT) regions. A few foci of NFD were present in the hypothalamus (HT) and in the midbrain, including

TABLE 2. Time sequence studies; distribution of Bcl-2, Bax, oxidative stress products, redox-active iron and apoptosis in CA1 of the superior hippocampus in Al- and maltol-treated young and aged rabbits at 3, 24 and 72 hours.

Age	Treatment	Superior Hippocampus (CA1)														
		Bcl-2			Bax			Oxidative Stress			Redox Iron			Apoptosis		
		3hr	24hr	72hr	3hr	24hr	72hr	3hr	24hr	72hr	3hr	24hr	72hr	3hr	24hr	72hr
8 mo	Al-maltolate (n = 6)	+	++	+++	+	-	-	-	-	-	+	+	+	-	-	-
8 mo	Maltol (n = 6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4 - 5 yrs	Al-maltolate (n = 6)	+++	++	-	-	++	+++	+	++	+++	+	++	+++	-	+	+++
4 - 5 yrs	Maltol (n = 6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

See Materials and methods for details.
- = Absent or rare.
+ = Few.
++ = Moderate.
+++ = Many.
n = Number of rabbits.
maltol = Maltol-treated controls.
Data for rabbits sacrificed at 12 and 48 hrs not shown.

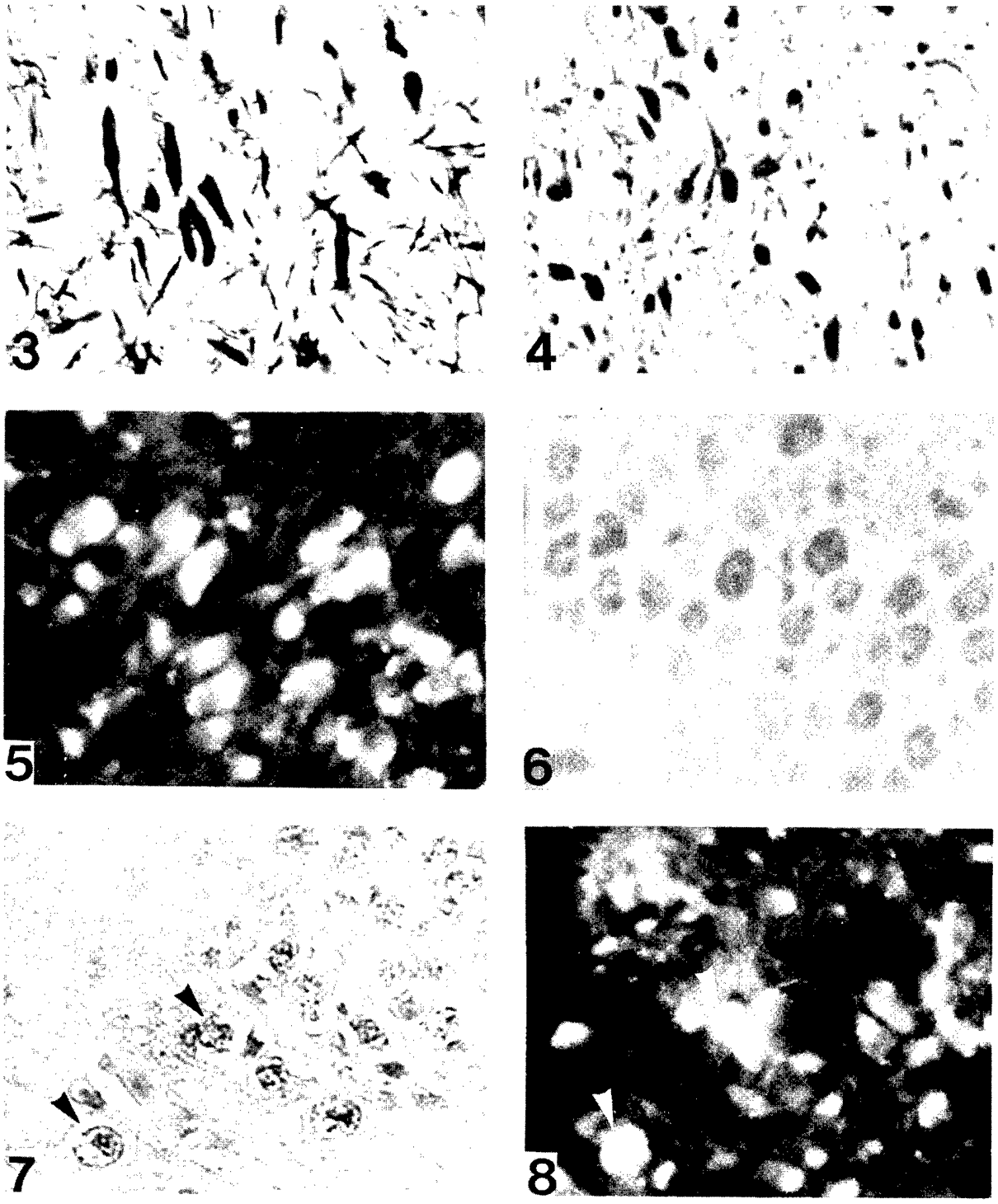
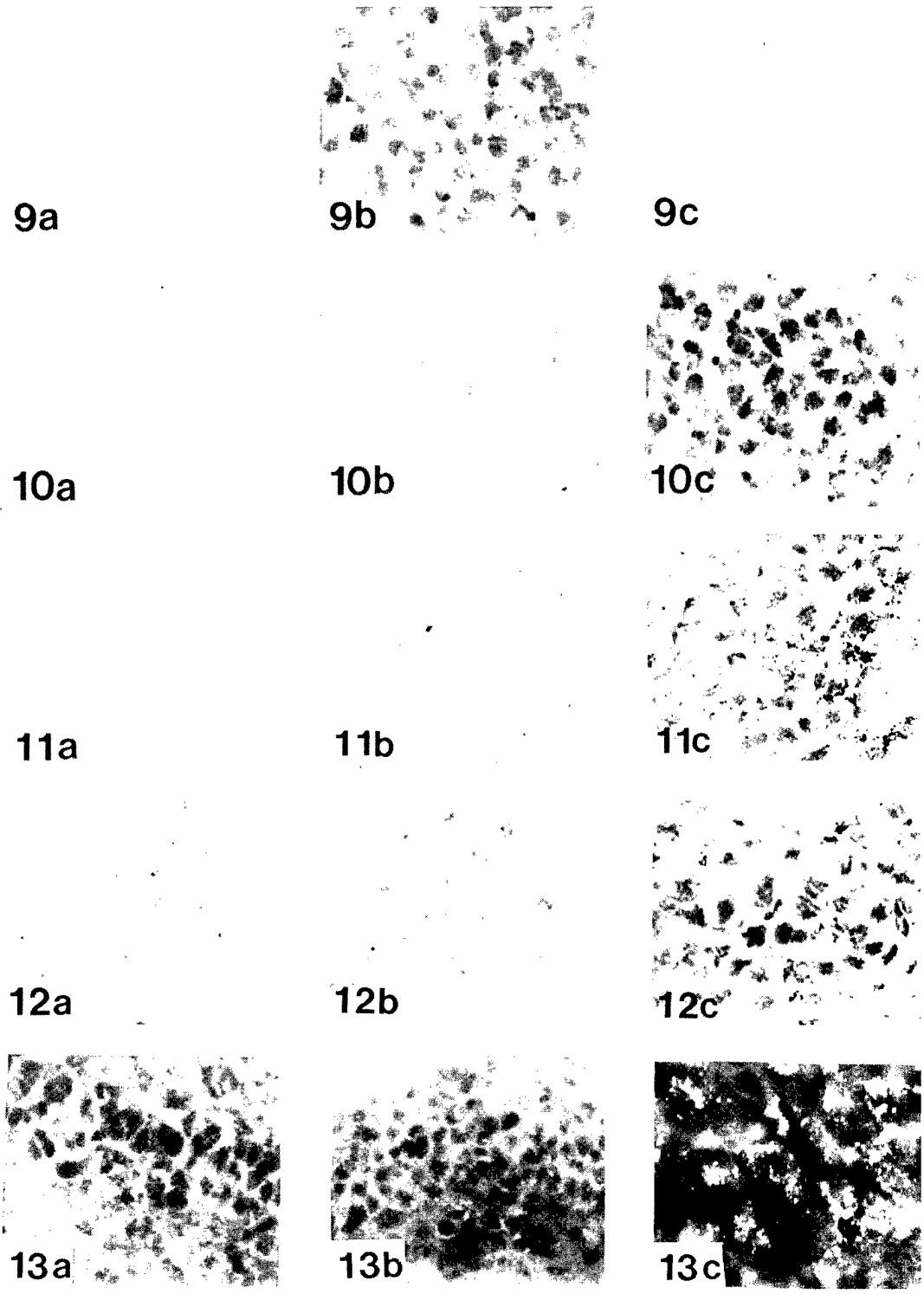


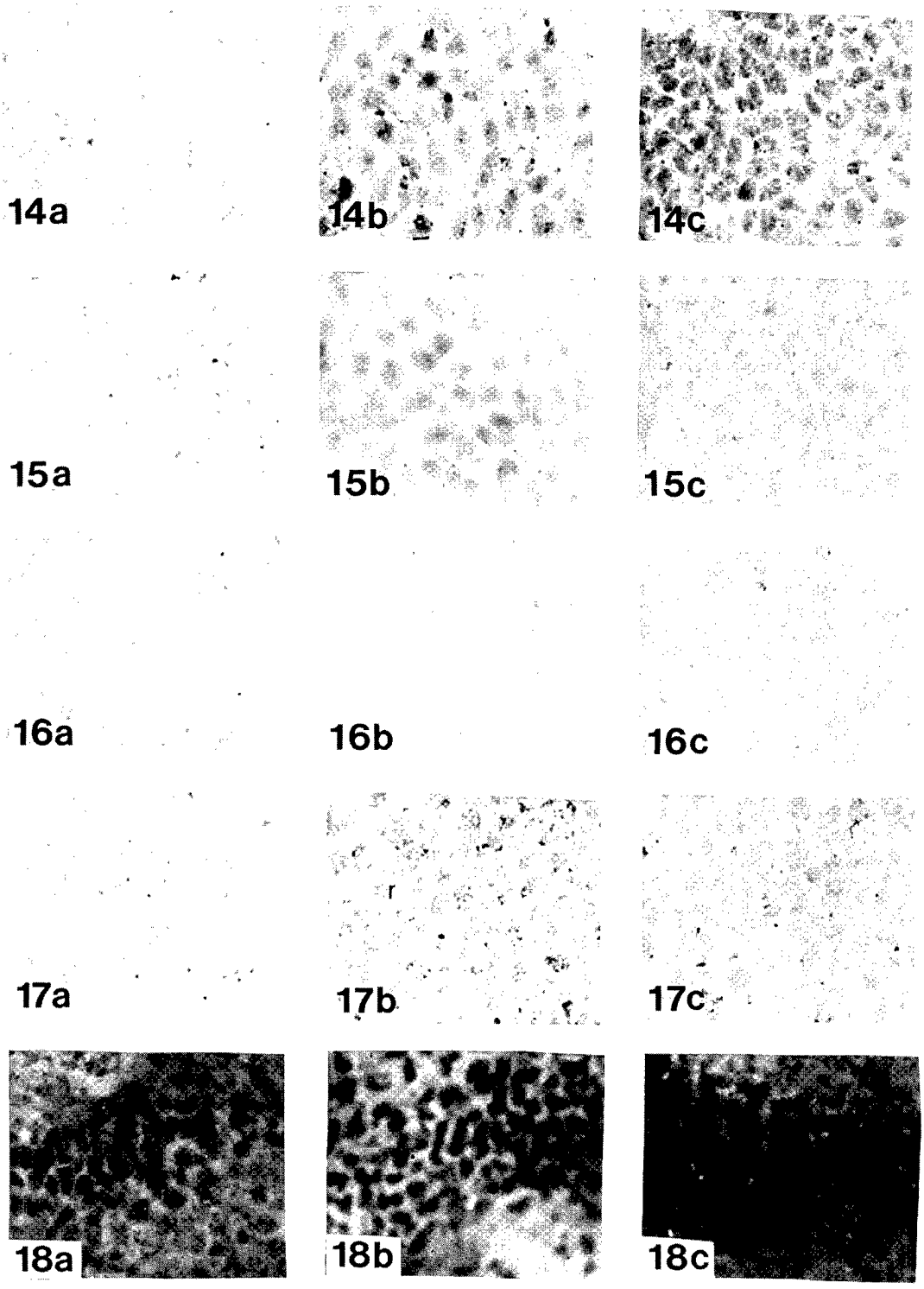
FIG. 3-8. Photomicrographs obtained from paraffin-embedded sections at an initial magnification of 400x: All animals were Al-treated and were 4-5 years old except that in Fig. 6 which was young (8 months).

FIG. 3. Silver-positive aggregates (NFD) in pyramidal neurons of the inferior segment of hippocampus (IH). Bielschowsky's method for silver impregnation. **FIG. 4.** PHF-1 immunopositivity of NFDs in the same region and animal as in Figure 3. Lightly counterstained with hematoxylin. **FIG. 5.** Apoptosis in pyramidal layer (CA1) of the inferior segment of hippocampus in an Al-treated aged rabbit; apoptosis is absent in the same region in a young treated rabbit **(FIG. 6)**. TUNEL technique, fluorescence detection. **FIG. 7.** Oxidative stress reaction product (arrowheads) in pyramidal layer of the inferior segment of hippocampus. Not counterstained, standard light microscopy. **FIG. 8.** Colocalization of apoptosis with oxidative stress in the same neurons. Same techniques as above. Fluorescence detection.



FIGS. 9-18. Photomicrographs of neurons in the pyramidal layer (CA1 region), superior segment of the hippocampus, in aluminum (Al)-treated (at 3 and 72 hours) or in maltol-treated control rabbits (at 72 hours). In each figure, a) is the maltol-treated control and b) and c) are Al maltolate-treated. The rabbits are either 4-year-old (aged) or 8-month-old (young) females. All micrographs are obtained from 14 µm-thick frozen sections at an initial magnification of 400x and are not counterstained. All are standard light micrographs, except for Figs. 13 and 18 which are fluorescent images.

Aged animals. **FIG. 9.** a) Absence of Bcl-2 immunopositivity in control; b) presence of marked Bcl-2 immunopositivity at 3hrs after Al maltolate treatment; c) minimal reactivity at 72 hrs after Al treatment. Diaminobenzidine (DAB) development. **FIG. 10.** Aged. a) Absence of Bax immunopositivity in control; b) faint positivity of Bax expression at 3 hrs; c) robust immunostaining at 72 hrs. DAB. **FIG. 11.** Aged. a) Absence of oxidative stress reaction products (carbonyls) in control; b) mild immunopositivity for oxidative stress at 3 hrs; c) marked reactivity at 72 hrs. DAB. **FIG. 12.** Aged. a) Absence of redox iron in control; b) presence of redox iron at 3 hrs; c) markedly increased reactivity at 72 hrs. DAB. **FIG. 13.** Aged. a) Absence of apoptosis in control and at 3 hrs after Al (b); c) presence of apoptosis in numerous cells at 72 hrs. Fluorescence microscopy.



Young animals. **FIG. 14.** a) Absence of Bcl-2 in control; b) presence of Bcl-2 at 3 hrs after Al, increasing by 72 hrs (c). DAB. **FIG. 15.** Young. a) Absence of Bax in control; b) slight Bax immunopositivity at 3 hrs which is diminished at 72 hrs (c). DAB. **FIG. 16.** Young. a) Absence of oxidative stress in control and in Al-treated rabbits at 3 hrs (b) and 72 hrs (c). DAB. **FIG. 17.** Young. Absence of redox-active iron in control (a) with slight reactivity at 3 hrs (b) and, to a lesser extent, at 72 hrs (c). DAB. **FIG.18.** Young. Absence of apoptosis in control (a) and after Al-treatment at 3 hrs (b) and 72 hrs (c). Fluorescence.

the nucleus nerve occulomotorius (NNO) and nucleus ruber (NR) (Fig. 2a). Examples of the silver-positive NFD are shown in Fig. 3 (pyramidal layer of the hippocampus, inferior segment). Using mAb PHF-1, we found robust positivity of the NFD in the inferior segment of hippocampus (Fig. 4) and in cerebral cortical neurons of aged Al-treated rabbits. This immunostaining was absent in saline-treated controls (Table 1). Previous reports on this rabbit system have demonstrated that intracisternal Al administration induces NFD most strikingly in the medulla and upper spinal cord (Savory *et al.*, 1996; Muma *et al.*, 1996; Gaytan-Garcia *et al.*, 1996). However, in the present study, sectioning of the entire cerebrum was performed, from the frontal pole to the occipital pole, as well as sectioning of the brainstem, thus providing more detailed information on the distribution pattern of NFD in regions which are often significantly involved in Alzheimer's Disease pathology.

Oxidative Stress. Oxidative stress products were found in both segments of the hippocampus and in the superior cortex of the aged Al-treated rabbits but not in the subiculum (Table 1). In the superior hippocampus, oxidative induced carbonyls were observed on nuclear and cytoplasmic membranes and in the cytoplasm of pyramidal neurons. In contrast, oxidative stress products could not be detected in young Al-treated or control rabbits of either age (Table 1). In the inferior hippocampus (Fig. 7) and superior cerebral cortex, evidence of oxidative stress was additionally noted in neuronal nuclei and in neurites, sometimes associated with NFD in the neuronal perikarya.

Detection and Quantitation of Apoptosis. Apoptosis was observed in CA1 of the superior and inferior hippocampal segments, and in the superior cortex of the Al-treated animals (Fig. 2b). It was also present in the superior cortex of the Al-treated animals and, to a much lesser extent, in aged controls (Fig. 2b). All other regions examined and listed in Fig 2a were almost entirely negative, as were the aged saline-treated controls in these areas. Quantitative assessment of neurons exhibiting apoptosis in these 4 brain regions is shown in Fig. 2b; the levels of significance of these findings is given below. An example of the increased density of apoptotic cells is demonstrated in a section of CA1 in an aged rabbit (Fig. 5). This is compared to minimal evidence for apoptosis in the same region of a saline-treated control (Fig. 6).

Colocalization of Oxidative Stress and Apoptosis in the Same Neurons. Double-staining for oxidative stress and apoptosis demonstrated colocalization of these two events in pyramidal neurons of the inferior (Figs. 7 and 8) and superior segments of hippocampus, and in the superior cerebral cortex (not illustrated).

Statistical Analyses of NFD and Apoptosis. NFD formation (Fig. 2a) and apoptosis Fig 2b) were present in statistically significant numbers in Al-treated aged animals as compared to saline-treated or Al-treated young animals (same Figures). The major findings are as follows: (i) NFD formation in the SC, LC, IC, SPS, SH, IH, NA, and NLDT regions of brain in Al-treated young rabbits was not statistically significant when compared to the absence of NFD in the above regions in saline-treated young rabbits, while NFD in the above regions in Al-treated aged animals was statistically significant ($p<0.001$) when compared to the absence of NFD in brain regions in the saline-treated aged; (ii) NFD in the above brain regions in Al-treated aged rabbits was significant ($p<0.001$) when compared to similar regions in Al-treated young; (iii) the presence of apoptotic cells in SH(CA1), IH(CA1), and SC brain regions in Al-treated young rabbits was significant ($p<0.001$), except in LC, when compared to the absence of apoptosis in brain regions in saline-treated young. In Al-treated aged rabbits the presence of apoptosis in the above brain regions (including LC) was significant ($p<0.001$) when compared to saline-treated aged; (iv) the presence of apoptosis in SH(CA1), IH(CA1), SC and LC brain regions in Al-treated aged rabbits was significant ($p<0.001$) when compared to Al-treated young.

Time-Sequence Studies of Bcl-2:Bax, Oxidative Stress Products, Redox-Active Iron and Apoptosis in CA1 of the Superior Hippocampus at 3, 12, 24, 48 and 72 Hours after Al

Aged Rabbits. All *maltol-treated aged controls demonstrated negative reactivity* for Bcl-2 (Fig.9a), Bax (Fig. 10a), oxidative stress (Fig. 11a), redox-active iron (Fig. 12a) and apoptosis as represented by DNA fragmentation (Fig. 13a). *Following Al treatment* the pattern of *Bcl-2* staining was that of intense reactivity at 3 hours (Fig. 9b), decreasing through 24 hours, and attaining near-control levels at 72 hours (Fig. 9c). *Bax* had an opposite trend, showing negative staining at 3 hours (Fig. 10b), slight positivity at 24 hours (not shown), and intense immunoreactivity by 72 hours (Fig. 10c). Evidence of mild positivity for *oxidative stress* in Al-treated animals was seen by 3 hours (Fig. 11b), increasing through 24 hours to strong positivity at 72 hours (Fig. 11c). A similar pattern was observed for *redox-active iron accumulation* (Figs. 12b and c). *Apoptosis* was positive at 72 hours (Fig. 13c) but negative at 3 hours (Fig. 13b).

Young Rabbits. Young rabbits exhibited a much less intense response to Al treatment. As was found in control aged rabbits, all *maltol-treated young controls were negative* and are illustrated as follows: Bcl-2, Fig.14a; Bax, Fig. 15a; oxidative stress, Fig. 16a; redox-active iron, Fig. 17a; and apoptosis, Fig. 18a. By 3 hours in the *Al-treated young*

group there was some Bcl-2 reactivity (Fig. 14b), which was less that that in the Al-treated aged (Fig. 9b). However, *by 72 hours* in the Al-treated young group, Bcl-2 increased in intensity (Fig. 14c), in contrast to essentially control levels in the Al-treated aged (Fig. 9c). *Bax* was slightly positive at 3 hours following Al administration in the young animals (Fig. 15b), but was negative by 72 hours (Fig. 15c), again contrasting with the Al-treated aged animals (Figs. 10b and c). No evidence of *oxidative stress* was found in young animals at either 3 hours after Al (Fig. 16b) or at 72 hours after Al (Fig. 16c). *Redox-active iron* was slightly positive in the Al-treated young animals at 3 hours (Fig. 17b) and at 72 hours (Fig. 17c). No evidence of *apoptosis* was detected in the Al-treated young group at either 3 hours (Fig. 18b) or 72 hours (Fig. 18c). These findings are summarized in Table 2.

DISCUSSION

Several neuropathological changes occur in AD, suggesting that the disease is possibly multifactorial, since it is characterized by the formation of abnormal phosphorylation of tau and the aggregation of this protein into neurofibrillary tangles, and by the deposition of A_β, (Iqbal *et al.*, 1994; Su *et al.*, 1996). Other proteins, particularly α_1-antichymotrypsin and ubiquitin, are abnormally expressed or processed in AD (Iqbal *et al.*, 1994; Abraham *et al.*, 1990; Lowe *et al.*, 1993). These features of AD have been recognized for a few years, although recent reports on the brain from patients with this disorder have suggested that other abnormalities also occur and perhaps precede these classical features. One theme that has been discussed frequently is that of oxidative stress occuring as an important early change in the neurodegenerative process (Su *et al.*, 1997), leading to apoptosis, and perhaps constituting the most important event in neuronal death (Cotman *et al.*, 1996; Markesbery 1997). Specific regions of the brain are highly susceptible to the formation of neurofibrillary tangles (Terry 1994; Price *et al.*, 1991; Arnold *et al.*, 1991; Bobinski *et al.*, 1997). Also, the enhanced susceptibility of neurons in aged individuals to neurodegeneration is obviously important but has received little attention because of the lack of a suitable animal model for such investigations.

Using the Al-rabbit system we now show that several important changes found in AD can be closely reproduced. Aging of the animals has a pronounced effect on rendering neurons in certain brain regions susceptible to Al-induced neurofibrillary aggregates (NFD), in areas also affected in AD. These NFD typically develop in Al-treated aged rabbits in the cerebral cortex, hippocampus, thalamus and midbrain, all regions of the brain that are frequently involved or that may be involved in AD. The neurofibrillary aggregates are argyrophilic and are immunostained with mAbs recognizing the abnormal tau present in the neurofibrillary tangles of AD. Young adult rabbits treated with Al form only a few of these intraneuronal lesions.

Interestingly, a pattern of oxidative stress and apoptosis generally colocalizes with NFD in the aged animals, and is of significance in that this occurs in regions affected in AD. However, there are exceptions to this pattern. The subiculum, which may be affected in AD, is a site for the formation of many NFD in Al-treated aged rabbits, but is largely negative for oxidative stress and apoptosis. The significance of regional differences in the vulnerability to these pathological changes is unclear at the present time.

Our working hypothesis is that oxidative stress represents an important early event in the neurodegenerative process in Al-treated rabbits and in AD and related diseases. We propose that aging is a predisposing factor in determining the extent of the injury and whether recovery occurs, with apoptosis being an important part of the process. The response of proteins of the Bcl-2 and caspase families, members of which function as pro-apoptotic and others as anti-apoptotic proteins, are obviously important in determining if a neuron survives or dies. Whether the defect in aging is related to such responses in an attempt to control apoptosis, or whether increased oxidative stress occurs in aged neurons, perhaps resulting from impaired mitochondrial function, is unclear.

In the present report we have shown that the pro-apoptotic protein Bax increases in Al-treated aged rabbits as the anti-apoptotic Bcl-2 reactivity diminishes, and that these changes correspond to the development of extensive apoptosis. Young treated animals (which had rare or no evidence of apoptosis) have an ever-increasing Bcl-2 response, with minimal Bax immunopositivity. This observation is in accordance with the rare or absent evidence for oxidative stress at 3 hours in the young treated and control animals, as compared to that observed in the aged treated animals.

Studies in AD patients suggest that apoptosis may occur before neurofibrillary tangle formation (Su *et al.*, 1997) and that oxidative damage (Smith *et al.*, 1996) and redox-active iron accumulation (Smith *et al.*, 1997) are observed, presumably also as early events. In the present report we have demonstrated that these changes occur quickly after Al treatment and are associated eventually with neurofibrillary pathology. Of importance is the influence of aging, wherein these alterations in Al-treated rabbits are much more pronounced in the aged as compared to young animals.

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696 CYCLOSPORIN A PREVENTS CYTOCHROME C RELEASE IN AL-TREATED AGED RABBIT BRAIN

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We have previously shown that Al maltolate administered intracerebrally to aged rabbits induces intraneuronal neurofilament accumulation,together with biochemical and immunohistochemical changes suggestive of apoptosis. However,the mechanism by which aluminum may induce apoptosis is unclear. Mitochondrial changes represent a primary event in apoptotic cell death, and Al-induced oxidative stress may be linked to its direct action on mitochondria. Cytotoxic stimulation of mitochondria can result in a loss of mitochondrial membrane potential, and the release of cytochrome C from its position between the inner and outer mitochondrial membrane, into the cytoplasm. The appearance of cytochrome C in the cytosol is a fundamental event in the activation of the cell death cascade. We have tested the hypothesis that Al-induced oxidative stress is linked to cytochrome C release, and that pretreatment with cyclosporin A, which prevents alterations in the mitochondrial membrane potential, inhibits the translocation of cytochrome C. Aged female New Zealand white rabbits were injected intracisternally with either 250 μg cyclosporin A (in 100 μL) or 100 μL of saline. One hour later we injected 100 μL of 25 mM Al maltolate or saline, also via the intracisternal route. Animals were sacrificed at 3 hours following the second injection. Western blots showed that in the saline-Al-treated animals, cytochrome C immunoreactivity was present in both the cytoplasmic and mitochondrial fractions of cerebral cortex and hippocampus. In cyclosporin A pretreated animals, cytochrome C immunoreactivity was selectively and dramatically decreased in the cytosolic fractions of both the cortical and hippocampal samples. These findings implicate mitochondrial injury, as assessed by cytochrome C release, as an early event in Al-induced neuronal injury and also implicate the opening of the permeability transition pore as an important component of this process. Since the Al-rabbit model system simulates many of the biochemical and neuropathological changes seen in Alzheimer's disease and related neurodegenerative disorders, we propose that such processes of mitochondrial injury may be important early events in these human diseases.

697 TETRACYCLINE-CONTROLLED EXPRESSION OF PRESENILIN-1 IN TRANSGENIC MICE

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We sought to characterize several aspects of presenilin-1 function in the adult brain using lines of mice trangenic for either wild-type or mutant(PS1ΔE9) presenilin-1. In order to more easily manipulate the timing of transgene expression, we have constructed mice in which the human presenilin-1 gene is expressed under the control of a promoter that can be regulated by tetracycline. These animals co-express the Tet-transactivator protein under the control of the mouse prion protein promoter. Ideally, we would like this transgene to be the only form of presenilin present in the adult mice, so that expression of presenilin could be completely eliminated by administration of antibiotic. We are currently testing the ability of both wild-type and mutant tetracycline-controlled presenilin transgenes to rescue the developmental lethality that occurs by embryonic day 19 in presenilin-null mice. We are simultaneously characterizing the extent to which orally-administered tetracycline inhibits synthesis of transgene product in adult transgenic mice. These preparatory stuides should establish our ability to control presenilin expression through development and into the adult animal. Initial experiments will seek to characterize the cellular and molecular effects of presenilin-1-depletion in the adult brain. Future studies will explore the result of presenilin inactivation on amyloid deposition and plaque maturation in mice doubly transgenic for mutant human presenilin and humanized amyloid precursor protein.

698 TAU PROTEIN (τ) KINASES IN THE RAT HEAT SHOCK MODEL: IMPLICATIONS FOR ALZHEIMER'S DISEASE

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We have previously shown that heat shock induces rapid dephosphorylation of τ followed by hyperphosphorylation in female Sprague-Dawley rats. In this study, we analysed the activities of the proline-directed kinases Mitogen-Activated Protein Kinase [MAPK(42/44)],c-Jun NH2-terminal Kinase (JNK), Cyclin dependent kinase 5 (Cdk5), and Glycogen Synthase Kinase-3β (GSK-3β), and the non-proline-directed Ca^{++}/calmodulin dependent protein kinase IIα (CaMKIIα) at 0 ($n = 5$), 3 ($n = 4$), 6 ($n = 5$), 12 ($n = 5$), and 24 ($n = 5$) h after heat shock (42°C for 15 min) and in non-heat shocked controls ($n = 5$). Immunoblots of forebrain homogenates using phospho-specific antibodies directed against activated kinases showed overactivatio at 3 and 6 h after heat shock of MAPK(42/44), JNK, and CaMKIIα but not GSK-3β. Immunoprecipitation initial rate kinase assays of MAPK(42/44), JNK, and Cdk5 using their specific substrates (myelin basic protein, GST-cJun, and histone-H1, respectively) showed overactivation of all three kinases. However, when purified native bovine τ was used as substrate only JNK and Cdk5 but not MAPK(42/44)hyperphosphorylated τ at 3 and 6 h after heat shock. Kinase assays for GSK-3β and CaMKIIα as well as analysis of the activity of Protein Kinase A (PKA) are in progress. Because τ is abnormally hyperphosphorylated in Alzheimer's disease, these preliminary data suggest that JNK and Cdk5 may play a role in its etiopathogenesis. We also envision, when our studies are completed, the rat heat shock model as a useful mammalian model to test some treatment protocols for Alzheimer's disease.

699 SYNAPTOSOMES FROM APOLIPOPROTEIN E-DEFICIENT MICE ARE VULNERABLE TO OXIDATIVE INSULT FROM Aβ

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Apolipoprotein E (Apo E) plays an important role in the response to central nervous system injury. Compared with wild type (WT) animals, mice deficient in Apo E (KO) have increased markers of oxidative stress under basal conditions and show increased neurodegeneration and neuron death following oxidative insult. Apo E KO mice display greater basal levels of tyrosine nitration and lipid peroxidation (isoprostane formation) compared with WT mice. Additionally, Apo E KO mice are more vulnerable than WT mice to stroke and head injury. We have investigated the role of Apo E in the maintenance of synaptic homeostasis. In this study, we report an increased vulnerability of Apo E KO synaptosomes to beta-amyloid (Aβ)-induced injury. At low levels of Aβ(1-40) [1μM], Apo E KO synaptosomes have more reactive oxygen species (ROS) than WT synaptosomes; with increasing Aβ concentrations [10-100μM], larger increases of ROS are generated in Apo E KO synaptosomes than in WT synaptosomes. Aβ-induced increases in mitochondrial calcium were to a greater extent in Apo E KO mice than were observed in WT mice. Further, Aβ caused a loss of mitochondrial membrane potential that was more severe in KO mice than in WT mice. Synaptosomal membranes isolated from Apo E KO mice are structurally different than those isolated from Apo E WT mice, as assessed by changes in lipid and protein mobility in Electron Paramagnetic Resonance spectroscopy studies. The structural alterations found in Apo E KO membranes are similar to changes induced by oxidative stress in WT synaptosomal membranes. In addition, levels of protein and lipid oxidation were analyzed in basal and Aβ-treated synaptosomes from Apo E WT and KO mice. These studies suggest that Apo E KO mice are more vulnerable to oxidative stress and may have implications to oxidative damage in CNS injury and Alzheimer's disease. Support: NIH.

Poster Presentation: Biomarkers III

700 "ERYTHROCYTE ANTIOXIDANT ENZYME ACTIVITIES IN ALZHEIMER'S PATIENTS AND OTHER DEMENTIA"

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The cause of patological changes in Alzheimers disease /AD/ is a distrubance in the equilibrium between the reactive forms of oxygen and the antioxidative mechanisms. One of the possible explanation of this oxidative stress is a perturbation in antioxidations, expecially enzymatic systems. The aim of our study was the assessment of basic antioxidant enzymes activity in ertyhrocytes of AD as compared with non-Alzheimer dementia patients and to find out if there was a possible connection of it with the stage of the disease and the patients age. The study covered 33 AD patients in different stages of the disease, 20 patients with vascular dementia and 20 patients with mixed dementia. The control group was composed of 18 persons with normal cognitive functions. All the patients were subjected to tests on superoxide dismutase - SOD-1 /by Misra and Fridovich's method/, on glutathione peroxidase /by Little and O'Brian's method/ and on glutatione reductase by spectrophotometric method /after Ellman/, for the first time prior to instituting therapy and then every four months during a year's treatment. In the treatment use was made of acetylocholinesterase inhibitors /Rivastigmine, Donepezil/ as well as vasoactive drugs /eg.Vinpocetine/. With a view of assessing the dynamics of the disease-apart from a routine psychiatric examination, psychometric tests were employed: MMSE, CGI /Clinical Global Impression/ and Hachinski's scale. A statistically significant increase /$p<0.05$/ was noted in the activity of peroxidase and glutathione reductase while there was a reduction in the activity of SOD-1 /$p<0.001$/ in the early stages of AD and a