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Detection of Mild Cognitive Impairment by CogHealth

by

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ABSTRACT

By using a computerized test battery, CogHealth, we examined 20 subjects with Mild Cognitive Impairment (MCI) and 30 age-matched normal controls. The diagnosis of MCI was based on the definition by Petersen as well as the criteria of CDR. For all CogHealth tasks, a significant reduction was noted, especially in the task of associative learning, in the MCI group compared with the controls. We therefore concluded that the CogHealth is a useful tool to differentiate MCI from normal individuals. As suggested previously that gradual decline in memory over the year is evident in MCI patient, we will focus our concern to evaluate follow-up data from cognitive normal patients, who visit memory clinic, for early detection of MCI.

Key word : Mild Cognitive Impairment (MCI), CogHealth, Early diagnosis

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はじめに

CogHealth はオーストラリアの研究者により開発された認知機能測定ツールで、特に Mild Cognitive Impairment(MCI)を検出するためのものである[5]。パソコン上で5種類のトランプ・ゲームを行い、応答速度を1000分の1秒の高感度で測定することができるシステムである。ゲームは単純反応時間(Simple Reaction: SR)、選択反応時間(Choice Reaction:CR)、作動記憶(Working Memory:WM)、注意分散(Divided Attention:DA)、連合学習(Associative Learning:AL)の5つの課題からなり、これらを約30分間で測定する。解析はそれぞれのゲームに対する応答速度、回答の正確さおよび一貫性など、およそ300項目のデータによって行われる。解析結果は被験者と同年齢の健常者と比較し、平均値が±1.3SDの範囲に入るものを正常として表す。これらはインターネット回線でオーストラリアからの送受信を通して、数分で解析結果を得ることができる。

CogHealth の認知機能スコアを定期的にモニターすることで、自覚症状などを伴わない軽微な認知機能低下傾向を検出することが可能である。CogHealth による測定は文化、言語、教育程度などにより影響されず、学習効果もないとされている[1,3]。

本ツールの提供は、CogState社(本社:オーストラリア、メルボルン市)により欧米およびオセアニア地域で既に始まっており、日本では株式会社ヘルス・ソリューションが提供している。今回

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の研究で用いたものは英語版であり、近く日本語版が使用可能となる予定である。

今回我々は地域調査で健常と判定された住民健常群と福岡大学病院通院中のMCI状態の患者(MCI群)にCogHealthを実施し、本テストが両群の鑑別に有用か否か検討した。

Ⅰ.対象

健常群として検討した住民は利根町住民で、年齢は66-82才(平均72.6才、SD:4.3)で、男性11名、女性19名、教育年数は平均10.7年(SD:2.5)であった。MCI群は福岡大学病院通院者であり、年齢は63-85才(平均74.7才、SD:6.1)で、男性8名と女性12名、教育年数は平均10.2年(SD:1.7)であった。年齢、性と教育年数に関する統計学的検定では両群間に有意差は認められなかった。本研究におけるMCIは,1996年のPetersenの定義[8)]に基づき,診察の結果,DSM-ⅣおよびNINCDS-ADRDA[6)]の診断基準により認知症がないと診断され,自身で物忘れの訴えがあり,CDR(Clinical Dementia Rating)[7)] 0.5と判定され,基本的なADLに障害のないこととした。被験者からは文書による同意を得た。また本研究は福岡大学および筑波大学倫理委員会の承認を得ている。

Ⅱ.方法

1.検査について

この研究のためにSONYのVAIO(OS:Windows XP)を用いた。パソコン上で使用するキーは“K”と“D”

の二つのキーのみで、右利き者と左利き者では“K”と“D”の役割は反対になる。ゲームがスタートすると、緑の背景画面に刺激トランプカードが出現し、各課題において被検者はどちらかのキー押し反応が要求される。

被検者は静かな部屋で椅子に座り、どのようなゲームなのか検査時と同じ条件で十分に説明を受け、課題練習を実施した後、本試行になる。

2.課題について

課題はルールの説明（最大約2分間）、練習（各タスクごとに要する時間は異なる）検査の3部構成となっている。順調に推移すると（1）から（4）までのそれぞれの課題は3分30秒から4分で終了し、課題（5）は約6分である。課題詳細は以下のようである。

（1）SR課題

画面の中心部に裏を向いているカードが現れ、一定の間隔で表を向く。その時できるだけ早く“K”を押すというものである。カードの刺激持続時間は最大3500msecで、この際、無回答もしくは不正解の場合は、それを示す聴覚的フィードバックがなされる。正解の場合は一切なされない。カードは無回答の場合は1500msec、回答のあった場合には2500msecの速度で順次呈示される。

練習で4回の適切なキー押しが得られると検査が開始される。必要とされる適切な試行回数は35回であり、それが得られたら終了する。

（2）CR課題

カードが赤なら“K”を、黒の時には“D”を押す。他は（1）と同様で、練習時の必要正答数は3

～4回で、本試行は30回の正解が得られると終了する。

（3）WM課題

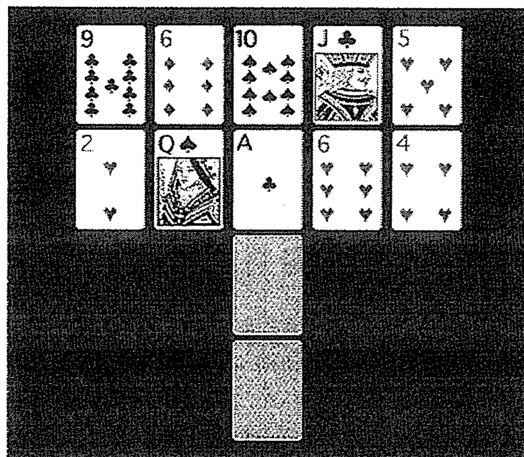
カードが直前に出たカードと同じ時には"K"を、異なる時には"D"を押す。カードの刺激持続時間は最大19000msecで、他は1）と同様である。本試行は30回の正解が得られると終了する。

（4）DA課題

画面の上下に1本ずつ白線があり、5つのカードがその白線の間を上下に無作為に動く。カードが上下どちらかの白線に触れたらすぐに"K"を押す。カードの刺激持続時間は最大5000msecで、この際、無回答もしくは不正解の場合は、それを示す聴覚的フィードバックがなされる。正解の場合は一切なされない。本試行は30回正しい反応が得られると終了する。

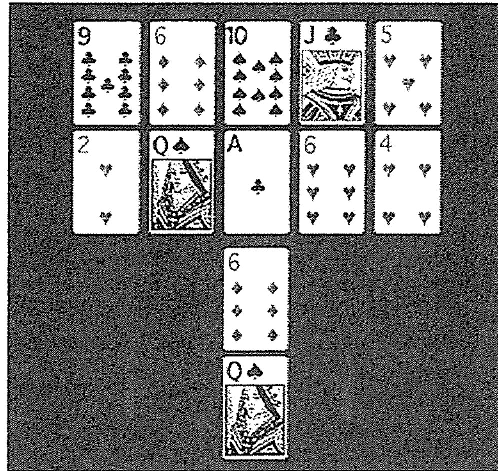
（5）AL課題

パソコンの画面の上方に、上下2枚で1組のトランプのカードが表向きで横に5つ並んでいる。その5組並んだ真ん中のカードの下に、裏向きの上下2枚で1組のカードがある。（図1）



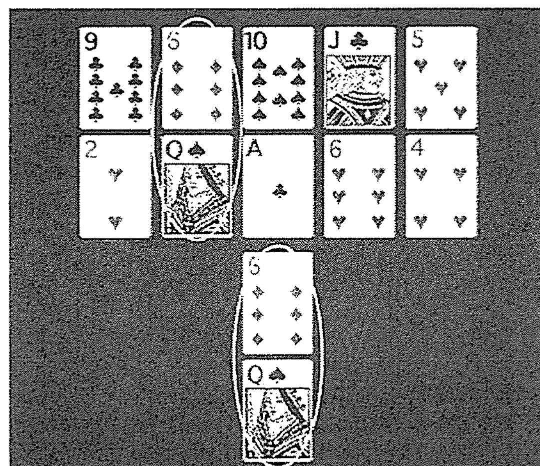
（図 1）

ゲームが始まると、その裏向きのカードが表に向く。上に並んだ5組のカードは最初から最後まで同じ組み合わせのままであるが、下に並んだ1組のカードは試行の都度、組み合わせが変わる。（図　2）



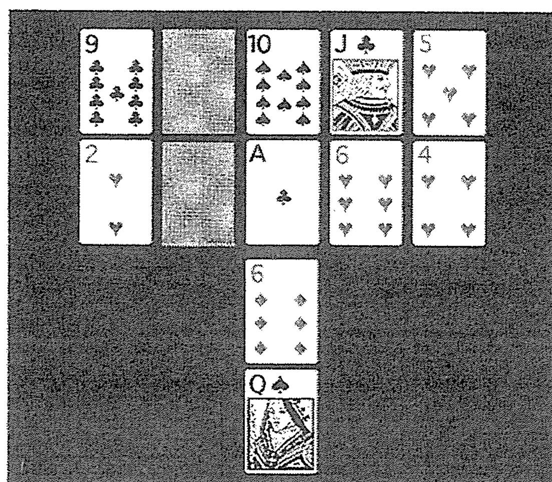
（図　2）

被検者はまず、下の1組のカードが裏面状態から表を向いた時、上に並んだ5組のカードのいずれかと同じ組み合わせのカードであるかどうかを判断し、キーを押す。（図　2，3）その場合、同じカードの組み合わせが出たら“K”を、違ったら“D”を押す。この時、カードの組み合わせは上下が逆になっていてもかまわない。（図　3）



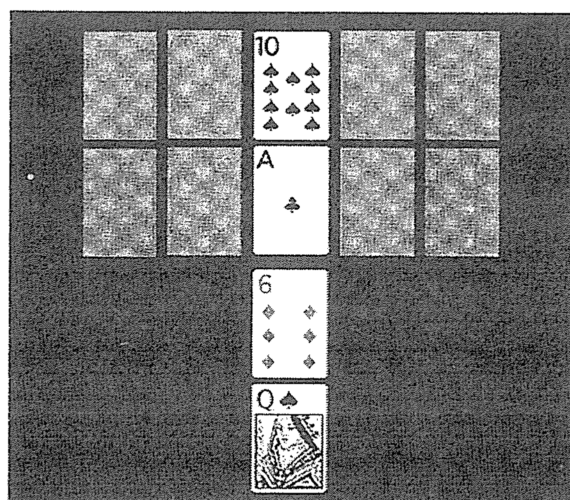
（図　3）

次に、上に並んだ5組のカードは、同じ組み合わせのカードが下に現れると、被験者が押したキーの正解、不正解に関わらず裏向きになる。（図　4）真ん中のカードのみ、同じカードが現れても最初から最後まで表を向いている。



（図　4）

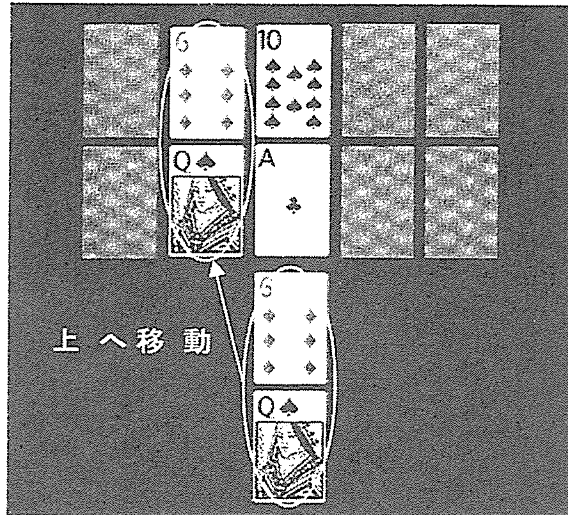
上に並んだ5組のカードの真ん中以外のカードが全て裏向きになると、次の段階に移る。（図　5）



（図　5）

すなわち被検者は裏向きになったカードの組み合わせを記憶しているかどうか判断するようにし

向けられる。上に並んだ5組のカードの真ん中以外が裏を向いてしまった状態で、下に現れたカードの組み合わせが、上に並んだ5組のカードの組み合わせのいずれかと同じものかどうかを判断する。（図 6）



（図 6）

次に、上に並んだ5組のカードと同じ組み合わせのカードが下に現れると、被験者の押したキーの正解、不正解に関わらず、上の同じ組み合わせのカードが表を向き、下のカードが上のカードの位置まで移動して重なり、すぐに裏向きに戻る。この時、カードの位置や組み合わせをカードの動きから再確認できるため、記憶していなかったカードを学習することができる。従ってカードの記憶学習ができているかどうかが判断できる。カードの刺激持続時間は最大 8500msec で、他は1）と同様である。この課題では50回の試行が得られると終了する。

（6）最初のゲームと同じゲームを最後にもう一度行う。35回の適切な試行が要求される。

以上の software は http://coghealth.com で自

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由に見ることができる。

課題（1）から（3）、（5）、（6）はゲーム開始時は1500msecの間カードが裏向きで固定され、その後から各課題において前述した通り刺激カードが画面上に登場する。この際、不正解のもの、反応できなかった場合や刺激後100msec以内の反応は自動的に解析から排除される。

Ⅲ．データの解析

各被検者の各課題について正解反応の数を計算し、全試行の％として表した（反応の正確さと定義する）。また各課題の正解の試行について平均反応速度（msec）（反応速度と定義する）と標準偏差値（SD）（反応の一貫性と定義する）を求めた。データは同年齢の健常被検者の平均値と標準偏差値をもとに、平均値を100で標準偏差値が10となるように正規化をおこない、数値として表現している。従って反応速度でも速度が速いものほど数値は大となる。

Ⅳ．結果

1．　MMSE得点

健常者群は平均29.0（SD：1.6）で、MCI群は平均25.9（SD：2.2）で、t-testにより有意差を認めた（$p<0.01$）。

2．　CogHealth得点

図7に各課題の反応速度、正確性と一貫性についての両群の項目別比較を示す。全ての課題でMCI群の成績が健常者群を下回った。反応速度においてはDAを除く全ての課題でMCI群の成績が健

常者群を下回った。一方、正確性においては AL についてのみ MCI 群の成績が健常者群に劣っていた。また一貫性においては全ての課題で MCI 群の成績が健常群に比べ、有意に劣っていた。

（図7挿入箇所）

3. MMSE と CogHealth 得点との相関

15項目全ての項目との間の重相関係数を求めると、0.713 と強い相関（P＜0.01）を認めた。次に CogHealth 各課題項目と MMSE 得点との相関を求めると表1に示すように、多くの項目で相関が見られるが、特に AL では全ての評価項目で有意な相関が認められた。SR では一貫性（P＜0.05）が、CR と WM では反応速度（p＜0.01）と一貫性（p＜0.05）が、DA では一貫性（P＜0.05）が、AL では全ての評価項目との間に有意な相関を認めた。

（表1挿入箇所）

4. 性別による差（図8）

性による差は AL にのみ認められその比較をみると、健常者群では男女間で統計的に有意差は見られないが、MCI 群では正確性と一貫性において、男性に比べて有意に女性の得点が低かった（P＜0.05）。

（図8挿入箇所）

5. 年齢との相関（表2）

健常者群では AL の正確性のみに有意な相関が見られたが、MCI 群では WM の3側面と DM の一貫性との間に認められた。

（表2挿入箇所）

V. 考察

本研究で行った CogHealth は、本来個人別のデ

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ータベースに基づき、健常高齢者を長期に亘って定期的に検査を行うことで短期記憶(瞬時記憶、作動記憶)、注意力(持続的注意力、選択的注意力、分割的注意力)を測定し、MCIの早期発見を目的として開発されたものである[5)]。成績評価は自動的に行われ、しかも短時間であることから健診施設などで大いに利用される可能性がある。

今回は臨床的に健常と判断された健常者群とMCI群に対して単純に1回目のデータを比較したものである。その結果、健常者群に比べMCI群では全ての課題で成績が低かった。特にALにおいては、全ての項目でMCI群の成績が有意に劣っていた。ALは短期記憶と注意力の両者を要求する課題であり、MCI状態に陥った被験者が最も苦手とする課題であると考えられる。このことは過去に行われたCollieらの報告と一致した[2)]。

CogHealthは測定結果のみならず、実際の検査現場でもMCI状態の被験者の教示に対する理解力から、記憶や注意力に問題のあることが容易に推測できた。SR、CRとWMではカードの提示状態が相似し、CR、WMとALでは色、数字とマークの判別を必ず“K”か“D”のいずれかのキー押しで要求されることから、MCI群の被験者は時折、課題のルールを混同した。特にCRとWMにおいてその傾向が強く、課題WM、ALにおいては教示内容を検査中に忘れてしまうことが多くみられた。

MCIは一年で10－15％がAlzheimer病に移行すると考えられている[8)]が、約50％は後の心理検査では改善するともいわれている[2,4)]。それ故Alzheimer病の前状態を意味する本当のMCIとは

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どのような状態か明瞭に示す検査診断法が求められている。

CogHealth を臨床現場に導入するために行った予備的な今回の研究により、健常者と MCI 群を本テストが明瞭に分離することが示された。従って３－６ヶ月毎に本テストを同一被験者に実施し、もしも経過とともに進行性の得点低下がみられ、かつ AL での明らかな成績低下が示されることは MCI 状態に陥ったと判断可能であろう。そしてこの時点から Alzheimer 病への進行予防の治療が行われるべきであろう。今後我々は、物忘れ外来等で臨床的に健常と判断された受診者の、CogHealth での得点変化を長期にわたり検討することで、CogHealth が MCI 状態への移行診断に寄与するか否かを検討する予定である。

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The Effect of Cholesterol and Monosialoganglioside (GM1) on the Release and Aggregation of Amyloid β-Peptide from Liposomes Prepared from Brain Membrane-like Lipids*

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In order to investigate the influence of cholesterol (Ch) and monosialoganglioside (GM1) on the release and subsequent deposition/aggregation of amyloid β peptide (Aβ)-(1–40) and Aβ-(1–42), we have examined Aβ peptide model membrane interactions by circular dichroism, turbidity measurements, and transmission electron microscopy (TEM). Model liposomes containing Aβ peptide and a lipid mixture composition similar to that found in the cerebral cortex membranes (CCM-lipid) have been prepared. In all, four Aβ-containing liposomes were investigated: CCM-lipid; liposomes with no GM1 (GM1-free lipid); those with no cholesterol (Ch-free lipid); liposomes with neither cholesterol nor GM1 (Ch-GM1-free lipid). In CCM liposomes, Aβ was rapidly released from membranes to form a well defined fibril structure. However, for the GM1-free lipid, Aβ was first released to yield a fibril structure about the membrane surface, then the membrane became disrupted resulting in the formation of small vesicles. In Ch-free lipid, a fibril structure with a phospholipid membrane-like shadow formed, but this differed from the well defined fibril structure seen for CCM-lipid. In Ch-GM1-free lipid, no fibril structure formed, possibly because of membrane solubilization by Aβ. The absence of fibril structure was noted at physiological extracellular pH (7.4) and also at liposomal/endosomal pH (5.5). Our results suggest a possible role for both Ch and GM1 in the membrane release of Aβ from brain lipid bilayers.

The pathology of Alzheimer's disease (AD)[1] includes extracellular amyloid plaques, intraneuronal neurofibrillary tangles, synaptic loss, and neuronal cell death. The major components of amyloid plaques are the amphiphilic 40 and 42 residue peptides, Aβ-(1–40) and Aβ-(1–42) (1, 2). Amyloid β-peptide (Aβ) consists of a hydrophilic N-terminal region (residues 1–28) and a hydrophobic C-terminal region (residues 29–40 or 29–42). The hydrophobic part of Aβ is originally part of a transmembrane α-helix of APP anchored in the membrane of several subcellular compartments, including the ER (3). Proteolysis by the enzyme(s) γ-secretase leads to the formation of Aβ within the membrane. Thus, the membrane release of Aβ following this enzyme cleavage should play a pivotal role in subsequent amyloid plaque formation.

Recent studies have shown that the interaction of Aβ and lipids plays an important role in the pathogenesis of AD. For instance, the fibrillogenic properties of Aβ are in part a consequence of the composition of the membrane in which it resides, its peptide sequence, and its mode of assembly within the membrane (4). In terms of membrane composition, Ch and GM1 in neuronal cell membranes are widely accepted to be modulators of membrane-associated Aβ fibrillogenesis and neurotoxicity (5, 6). The formation of GM1-bound Aβ, which is thought to be a seed for the formation of toxic amyloid fiber, depends on the concentration of Ch in model membranes prepared from GM1/Ch/sphingomyelin (SM) (7). Additionally, oligomeric Aβ can promote the release of lipids from asterocytes and neurons by forming Aβ-lipid particles consisting of Ch, phospholipids, and GM1 (8).

It has been suggested that Aβ-(1–42) is essential to the early development of AD pathology but is not alone sufficient to promote the formation of mature neuritic plaques unless it is succeeded by the deposition of Aβ-(1–40) (9). Compared with Aβ-(1–40), Aβ-(1–42) has been shown to have a greater potential for aggregation (10). Studies of Aβ-lipid interaction using total brain lipid extract have shown that the peptides interact in different ways: 1) Aβ-(1–40) destabilizes model membranes and 2) Aβ-(1–42) initially destabilizes but then with time proceeds to stabilize the membrane (11). These findings are consistent with a "seeding" hypothesis, in which the aggregates of Aβ-(1–42) act as an initiation factor for early plaque formation, which is then followed by the progressive accumulation of Aβ-(1–40) in the AD brain. So too, they provide an insight into a mechanism of fibrillogenesis, which is at least in part controlled by the release of Aβ from the membrane.

AD is a disease that involves attack of the central cerebral cortex. To investigate how Ch and the ganglioside GM1 may influence the release of Aβ-(1–40) and Aβ-(1–42) from cerebral cortex membranes we have prepared and examined some model-Aβ-containing liposomes. The liposomes are composed of a lipid-mixture similar in composition to cerebral cortex membranes (CCM-lipid). The liposomes prepared were of four different lipid compositions (see Table I); CCM-lipid, liposomes with no GM1 (GM1-free lipid), liposomes without cholesterol (Ch-free lipid), and liposomes without GM1 and Ch (Ch-GM1-

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[1] The abbreviations used are: AD, Alzheimer's disease; Aβ, amyloid β-peptide; APP, amyloid precursor protein; CCM-lipid, a lipid-mixture similar to the composition of cerebral cortex membrane; Cer, galactocerebroside; Ch, cholesterol; CD, circular dichroism; Me_2SO, dimethylsulfoxide; egg PC, egg yolk L-α-phosphatidylcholine; egg PE, egg yolk L-α-phosphatidylethanolamine; GM1, monosialoganglioside GM1; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; PS, L-α-phosphatidyl-L-serine; SM, sphingomyelin; TEM, transmission electron microscopy.

TABLE I
Composition of lipids in the peptide-containing liposomes of this study

	Cerebral cortex lipid[a]	CCM-lipid	Ch-free lipid	GM1-free lipid	Ch-GM1-free lipid
	w/w %	*mol %*			
PC	13.9	15.4 (14.0)	18.8	17.1	20.4
PS	8.0	8.9 (7.0)	13.3	9.8	11.7
PE	23.9	26.5 (24.0)	29.2	29.4	31.4
SM	8.0	8.9 (4.0)	12.5	9.8	11.7
Ch	13.3	14.8 (26.0)		16.3	
Cerebroside	14.3	15.9 (15.0)	16.6	17.6	21.0
GM1	8.75	9.7 (4.0)	9.6		
Plasmalogen	9.94				

[a] Values for CCM-lipid are those of Rossiter (Ref. 24).

free lipid). Liposomes were prepared by hydrating an organic film composed of a mixture of Aβ and one of the four lipid mixtures outlined in Table I. This method of liposome preparation was chosen to reflect a more natural release process as opposed to a method involving addition of Aβ to preformed liposomes.

The release of Aβ occurs from plasma, endosomal, lysosomal, and Golgi membranes by proteolysis (12–14). All experiments were done at a pH of 7.4 to represent a physiological extracellular pH. However, to model whether or not the more acidic environment of the endosomal pathway is significant we have carried out a number of our experiments at a pH of 5.5. The common endosomal and lysosomal pH values are thought to be 6.0 and 4.5–5.5, respectively (15, 16); consequently we chose 5.5 to be representative of both organelles. We report here the mode of interaction of Aβ-(1–40) and a mixture of Aβ-(1–40)/Aβ-(1–42) (10/1) with each of the four types of liposome as revealed by CD, turbidity, and negative-stained TEM measurements.

EXPERIMENTAL PROCEDURES

Lyophilized amorphous powders of synthetic Aβ-(1–40) and -(1–42) from dimethyl sulfoxide (Me_2SO) were obtained from Peptide Institute Inc. (Osaka, Japan), Purities were analyzed by HPLC and amino acid analyses. Egg yolk L-α-phosphatidylcholine (egg PC), egg yolk L-α-phosphatidylethanolamine (egg PE), bovine brain L-α-phosphatidyl-L-serine (PS), bovine brain galactocerebroside (Cer), bovine brain sphingomyelin (SM), and Ch were purchased from Sigma-Aldrich Japan K. K. (Tokyo). Bovine brain ganglioside GM1, 1.1.1.3.3.3-hexafluoro-2-propanol (HFIP), and other chemicals were purchased from Wako Pure Chemical Ind, Ltd. (Osaka, Japan).

It has been recognized that the self-assembly of Aβ is dependent on the initial Aβ structure, *i.e.* whether it is monomeric and has a random coil structure or whether it has a β-sheet structure (17, 18). In the present study, the lyophilized powders of the peptide(s) were dissolved in HFIP, a solvent well known for its good solubilizing and α-helical structure-promoting properties. In HFIP solution, Aβ is monomeric (19–21). The peptide stock solution was made by dissolving the lyophilized amorphous powder in HFIP. Each liposome, composed of the appropriate mixture of lipids and peptide, was prepared by hydration and sonication of a film obtained from the evaporation of a mixture of the lipids in $CHCl_3$ solution and Aβ in HFIP solution.

CD Spectrum Measurements—Mixed films of lipids and Aβ-(1–40) or Aβ-(1–40)/Aβ-(1–42) (10/1) in HFIP were prepared by evaporating the organic solvents under a stream of nitrogen. Any residual solvent in the film was removed *in vacuo* overnight. Films were hydrated in Tris buffer solution (5 mM Tris, 100 mM NaCl, pH of 7.4 or 5.5) and sonicated by ultrasonic irradiation in the cuphorn of a Branson Model 185 sonifier (Danbury, CT). Solutions were sonicated at room temperature for about 30 min until they became transparent. CD spectra were measured on a JASCO J-600 apparatus (JASCO, Tokyo, Japan) controlled by a personal computer (NEC PC-9801) using a 1-mm pathlength quartz cell at 25 °C. Four scans were averaged for each sample. An averaged blank spectrum (vesicle suspension or solvent) was subtracted from each sample spectrum. The peptide and lipid concentrations were 50 μM and 1 mM, respectively. Conformational analyses were performed using CONTIN3 in a software package for analyzing protein CD spectra (CDPro) via the Internet (22). In our experiments, the program has been successfully applied to the solutions of peptides consisting of α-helix, β-sheet structure, β-turn, and random structure such as the p53 tetramerization domains that have been well characterized by NMR (23).

Turbidity Measurements—A mixed solution of lipids in chloroform and Aβ in HFIP measured to the appropriate peptide/lipid mol ratio was placed in a round bottom flask and dried under a stream of N_2 gas. The residual films were further dried overnight *in vacuo* and then hydrated with Tris buffer saline solution (5 mM Tris, 100 mM NaCl, pH of 7.4 or 5.5) by vortexing and then sonicated as described above. Lipid concentrations were kept to a concentration of 100 μM in the same Tris buffer at 25 °C. The absorbance of the sample solution was recorded at 400 nm using a JASCO spectrometer (JASCO Corp., Tokyo, Japan) after vigorous vortexing.

Transmission Electron Microscopy (TEM)—Each sample was absorbed onto a carbon-coated copper grid (mesh) by floating a drop of sample solution. Excess solution was removed by filter paper blotting, the grid was washed by floating a drop of water, and then the water was removed. The sample on the grid was then negatively stained with an aqueous phosphotungstic acid (1.0%), and the excess staining solution was removed. After drying, the samples were imaged with a HITACHI HU-12A electron microscope (Hitachi, Japan) operating at 100 kV. Samples prepared for the CD experiments were also used for the TEM experiments. The peptide and lipid concentrations were 50 μM and 1 mM, respectively.

RESULTS

Compositions of Lipid Mixtures Similar to Cerebral Cortex Membrane—To investigate the roles of GM1 and Ch in Aβ-lipid interactions, we chose to model cerebral cortex membranes, because this is the main location of amyloid in the body. Values for the composition of total lipid extract from cerebral cortex membranes were those reported by Rossiter (24). Because of its easily oxidative property and its instability under acidic conditions, plasmalogen was not included in the model liposomes of this study. In the human cerebral cortex tissue, there is about 10% (w/w) of plasmalogen. We have adjusted the proportion of the other components to allow for this omission as shown in Table I, where we list the compositions of the four types of liposomes examined. Ch (26 mol %) is significantly more abundant in CCM membranes than GM1 (4 mol %).

Secondary Structure of Aβ-(1–40) in Lipid Bilayers—Amyloidogenesis involves a transition from random or α-helical to a β-structure, which is necessary for fibril formation *in vivo*. Thus we monitored the conformational change of Aβ-(1–40) or Aβ-(1–40)/-(1–42) (10:1, molar ratio) in the four liposome systems (Table I) by CD spectroscopy over a period of 7 days. In buffer solution, the peptide Aβ-(1–40) adopts a mainly random coil structure upon addition of the peptide stock solution to the buffer solution (Fig. 1*A*). This result agrees with the CD data reported previously (7, 25). Then, after 5 days, it takes a mainly β-sheet structure as suggested by the peak minimum around 218 nm. A conformational analysis of its spectrum showed 15% α-helix and 30% β-structure. This main structure still persisted after 7 days.

In the CCM membrane at pH 7.4 (Fig. 1, *B-a*), upon preparation from the lipid-peptide film, Aβ-(1–40) adopts a mainly random structure within the liposomes, however, after 1 day a

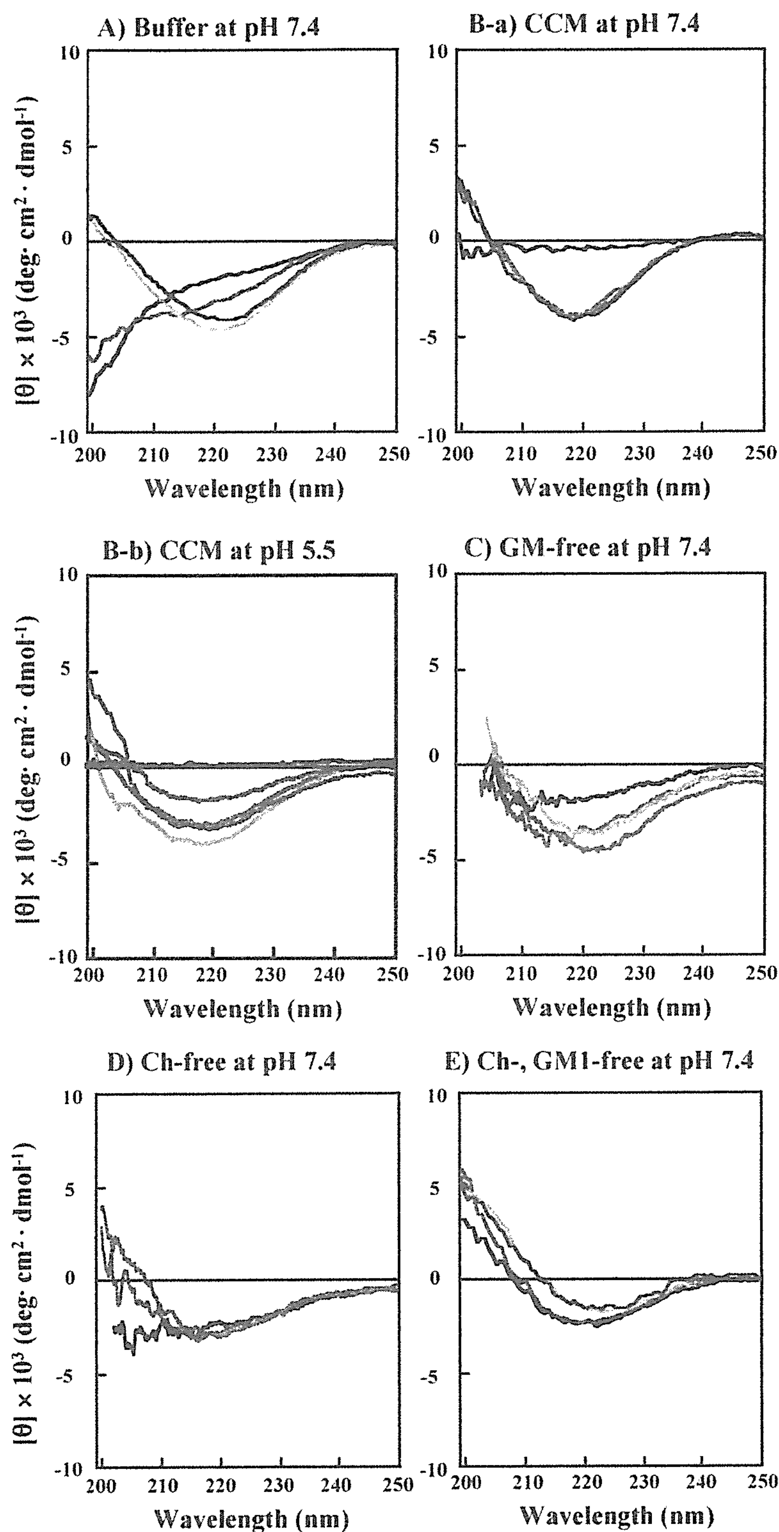

FIG. 1. **CD spectra of Aβ-(1–40) in Tris buffer (*A*), CCM at (*a*) pH 7.4 and (*b*) pH 5.5 (*B*), GM1-free (*C*), Ch-free (*D*), and Ch-GM1-free (*E*) liposomes.** Lipids over a period of 7 days after the incubation of peptides to liposomes are shown: immediately after preparation (*black*), at 1 day (*green*), 3 days (*red*), 5 days (*orange*), and 7 days (*blue*). Peptide and lipid concentrations are 50 and 1 μM, respectively.

broad negative band is seen around 218 nm, the intensity of which is approximately equal to that of the peak observed for the 7-day-old sample in buffer. But conformational analysis of the spectrum showed 60% β-structure and the same spectral pattern after 3, 5, and 7 days. When compared with the CD spectra in buffer, the β-structure formation was accelerated and more extensive. At pH 5.5, similar CD curves were obtained, though the rate of β-structure formation was significantly reduced. After 1 day under acidic conditions there was only 36% β-structure as compared with 60% at pH 7.4 (Fig. 1, *B-b*). On the other hand, the peptide in GM1-free lipid liposomes at pH 7.4 (Fig. 1*C*), showed a shallow negative band around 210 nm with 25% α-helix and 10% β-structure, and after 1, 3, and 5 days the negative band had shifted to 216 nm, corresponding to 45% β-structure and 10% α-helix, indicating that the absence of ganglioside slightly reduces formation of β-structure, compared with CCM membranes. Interestingly, in Ch-free membrane (Fig. 1*D*), Aβ-(1–40) had a negative band around 205 nm similar to that of GM1-free liposomes after preparation. Some α-helical structure (about 15%) was seen, but in time the band around 205 nm intensified, and a crossing point on the horizontal axis occurred characteristic of β-struc-

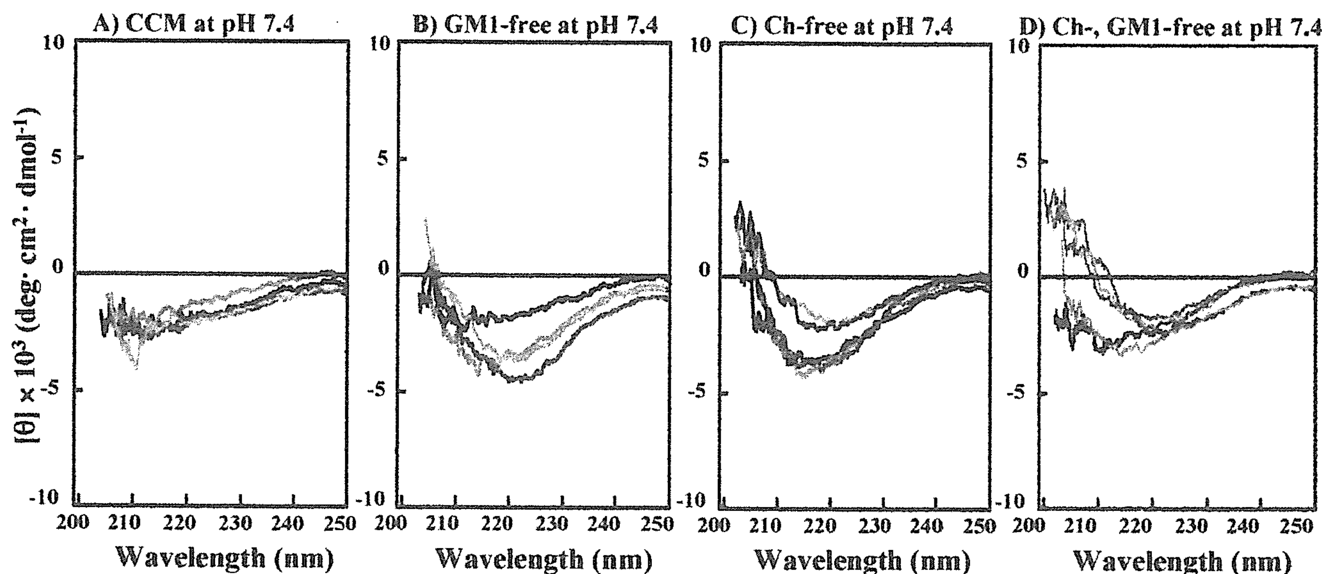

FIG. 2. **CD spectra of Aβ-(1–40)/Aβ-(1–42)(10/1) in CCM (*A*), GM1-free (*B*), Ch-free (*C*), Ch-GM1-free (*D*) over a period of 7 days after the incubation of peptides to liposomes.** Shown are spectra immediately after preparation (*black*), at 1 day (*green*), 3 days (*red*), 5 days (*orange*), and 7 days (*blue*). Peptide and lipid concentrations are 50 μM and 1 mM, respectively.

ture. After 3 days, β-structural content reached 50%, indicating, that in time, the absence of either Ch or the absence of GM1 led to a decrease in β-structure formation. For Ch- and GM1-free liposomes (Fig. 1*E*) at pH 7.4, Aβ-(1–40) showed a broad negative band around 220 nm upon preparation, there was some α-helical structure present. However, in time, the band shifted to 203 nm and became shallower, also the crossing point of the horizontal axis red-shifted to more than 210 nm, indicating decreased β-structure. In fact, the spectra could not be analyzed using the CDPro software (22). Similar CD spectra have been reported in which the spectrum has an atypical minimum at 223 nm and a maximum at 203 nm; this pattern is characteristic of amyloid fibers (26, 27). However, electron microscopy of Aβ-(1–40) did not detect any fibril structure in Ch-free or Ch-GM1-free membranes. These results indicated that the CD spectral patterns having the minimum at 223 nm and the maximum at 203 nm do not necessarily exhibit the characteristics of amyloid fibers. At pH 5.5, although the minima around 220 were shallower than those at pH 7.4, very similar CD curves were observed, and the fibril structure was not detected by TEM at either pH (data not shown). Consequently, it would appear that the presence of Ch and GM1 ganglioside is necessary for β-amyloid formation to occur.

CD Spectra of Aβ-(1–40)/Aβ-(1–42) (10:1)) in Lipid Bilayers—To investigate a seeding hypothesis in which aggregates of Aβ-(1–42) act as an initiation factor for early plaque formation, we examined changes in conformation of Aβ-(1–40)/-(1–42) (10:1, molar ratio) in the four model liposomes at pH 7.4 (Fig. 2) by circular dichroism spectroscopy. Our results show that in the CCM and Ch-free membranes, the rate of β-structure formation is greater than that for the corresponding Aβ-(1–40) liposomes. In CCM-lipid, upon preparation, we observed 40% β-structure which, after 1 day became 50%; for Aβ-(1–40) these values are 18 and 60% β-structure, respectively (Figs. 2*A* and 1*B*). In the GM1-free membrane, the CD spectral pattern of Aβ-(1–40)/-(1–42) is not different from that of Aβ-(1–40) (Figs. 2*B* and 1*C*). In Ch-free lipids, a mixture of Aβ-(1–40)/-(1–42) formed a β-structure more easily than the Aβ-(1–40) liposomes (Figs. 2*C* and 1*D*). Interestingly, in the Ch-GM1-free lipid liposomes upon preparation the peptide had a 35% β-structure and 10% α-helical structure. This was not observed for Aβ-(1–40) and in time it transformed the conformations to the other conformational mixture containing β-structure as described above (Fig. 2*D*). These results indicate that the rate of formation of the β-structure is generally promoted by the addition of Aβ-(1–40), although ultimately formation depends on lipid composition.

Turbidity Measurements—To monitor the aggregation or precipitation produced by lipid-peptide interactions, we measured the changes in turbidity in the buffer solution and solutions of the four Aβ-lipid mixture liposomes over a 7-day period (Fig. 3). Solution absorbance measured at 400 nm was plotted as a function of time. Days after successive film preparation, hydration, and sonication of mixture solutions for different ratios of Aβ-(1–40) and lipids are shown on the abscissa. The lipid concentration was kept at 100 μM. In the absence of liposomes, at a peptide concentration of 40 μM at pH 7.4, the absorbance gradually increased from 1 day and reached a maximum at 3 days, and then leveled out as a plateau (Fig. 3*A*). However, at lower peptide concentrations (5–20 μM), the aggregation behavior was dependent on peptide concentration. In conjunction with the CD results, it was concluded that increasing turbidity was attributable to β-structure formation, associated with Aβ aggregation.

For 40 μM Aβ in CCM liposomes at pH 7.4, solution turbidity gradually increased over 4 days, then it slightly decreased, but at lower peptide concentrations there was little change in turbidity (Fig. 3*B*). With the results of CD and TEM experiments in mind, little or no change in turbidity is due to an aggregation of Aβ-(1–40) to form a β-structure. Similar turbidity changes in CCM were obtained at pH 5.5 (date not shown). In the GM1-free liposomes at pH 7.4, solution turbidity did not change at the examined Aβ concentrations except for 40 μM peptide, where a slight decrease occurred 1 day after preparation and was followed by a gradual increase (Fig. 3*C*). Little or no change in turbidity was consistent with the CD and TEM results, which showed the formation of aggregated peptide-lipids particles (to be described below). Interestingly, in Ch-GM1-free lipid liposomes at pH 7.4, turbidities for 20 and 40 μM peptide decreased. For 40 μM peptide an especially dramatic decrease in turbidity occurred over the first day (Fig. 3, *E-a*). Similar changes in turbidity were observed at pH 5.5 (Fig. 3,

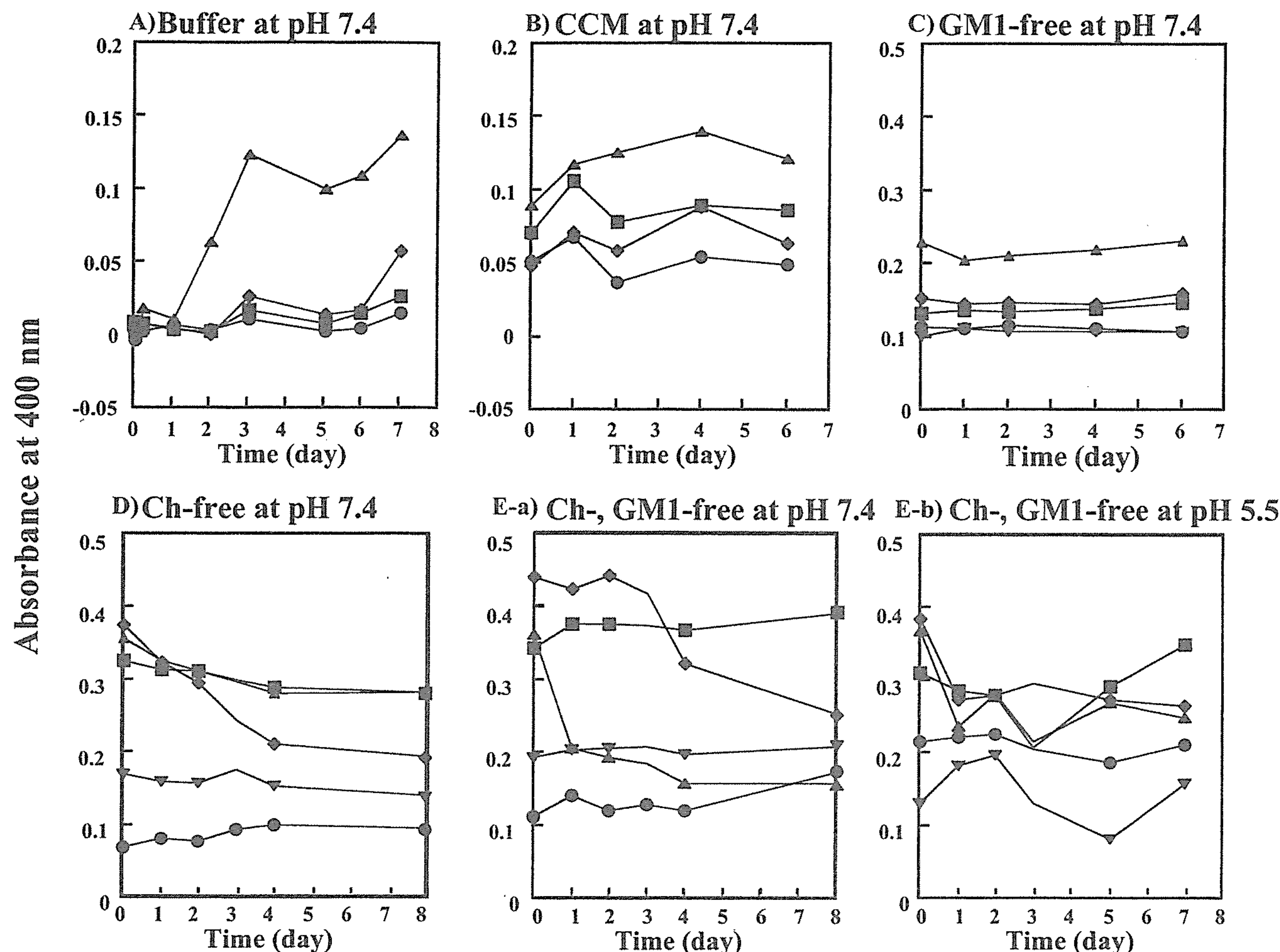

FIG. 3. **Changes in turbidity in buffer solution (*A*), CCM (*B*), GM1-free (*C*), Ch-free (*D*), Ch-GM1-free (*E*) at (*a*) pH 7.4 and at (*b*) pH 5.5 over a period of 7–8 days after the incubation of Aβ-(1–40) to liposomes.** Peptide concentrations are: 0 μM (●); 5 μM (▼); 10 μM (■); 20 μM (◆); and 40 μM (▲).

E-b). The decrease in turbidity indicated liposomes were solubilized by Aβ-(1–40), to form small peptide-lipid particles. The TEM image for this solution showed the liposomes became smaller with time and showed no fibril structure. Although a similar decrease in turbidity was observed for the Ch-free liposome solution (Fig. 3*D*), this was independent of peptide concentration; the turbidity of 20 μM Aβ was much larger than that of 40 μM. This also indicated that liposomes were solubilized and led to the formation of small particles.

Turbidity of Aβ-(1–40)/Aβ-(1–42) (10:1) was measured in CCM lipid and ChGM1-free lipid at pH 7.4 (data not shown). Initial turbidity did not depend on peptide concentration and did not change dramatically with time, which indicated the membrane solubilization did not occur as it did for Aβ-(1–40) in Ch-free and Ch-GM1-free membranes.

Studies by Transmission Electron Microscopy—To observe changes with time in the morphological characteristics of Aβ-(1–40) and Aβ-(1–40)/Aβ-(1–42) (10:1) both in buffer and in the liposomes we made measurements by TEM. Liposomes were prepared from a mixture of lipids (1 mM) and peptide (50 μM) and then examined over a period of 1–10 days under the electron microscope. The TEM samples were negatively stained. For Aβ-(1–40), after 1 day in buffer solution at pH 7.4, an indistinct fibril structure was observed, which after 10 days became better defined (Fig. 4*A*). In CCM lipid membrane at pH 7.4, a distinct fibril structure was observed, with a dark phospholipid membrane-like shadow after 1 day (Fig. 4*B-a*, *i*). After 10 days the same clear fibril structure seen in saline buffer was observed (Fig 4*B-a*, *iii*). At pH 5.5, lots of short filaments were visible after 1 day and clear long fibrils were observed after 3 days, indicating that the growth of fibril structure is slower than that at pH 7.4 (Fig 4*B-b*). This is consistent with the slower rate of β-structure formation seen by CD after 1 day at pH 5.5 relative to that at pH 7.4. In GM1-free membranes (Fig. 4*C*) at pH 7.4 after 1 day, long fibril structures were present along the surface of vesicles, which were probably large aggregated/fused liposomes. After 3 days, small vesicles emerged around the large aggregate liposomes, also some fibrils were still apparent along their surface. After 10 days we observed aggregates of small vesicles (several ten-fold nanometers in diameter). These results indicate that Aβ can disrupt the large liposomes into smaller vesicles, presumably by the formation of lipid-peptide complexes. After 1 day in the Ch-free membrane (Fig. 4*D*) at pH 7.4, a few relatively long fibrils were observed around the large liposomes. After 4 days, numerous small filament-like structures, probably lipid-peptide complexes, were present around the liposomes. Interestingly, after 10 days, thicker and longer fibrils (several ten-fold nanometers in diameter) were observed around the liposomes, which were different from the fibril structure observed in the buffer and CCM liposomes.

These results indicate that although the Ch-free membrane first releases some Aβ to create fibrils, in time, the membranes were slowly solubilized by Aβ to make short and thin fibrils and

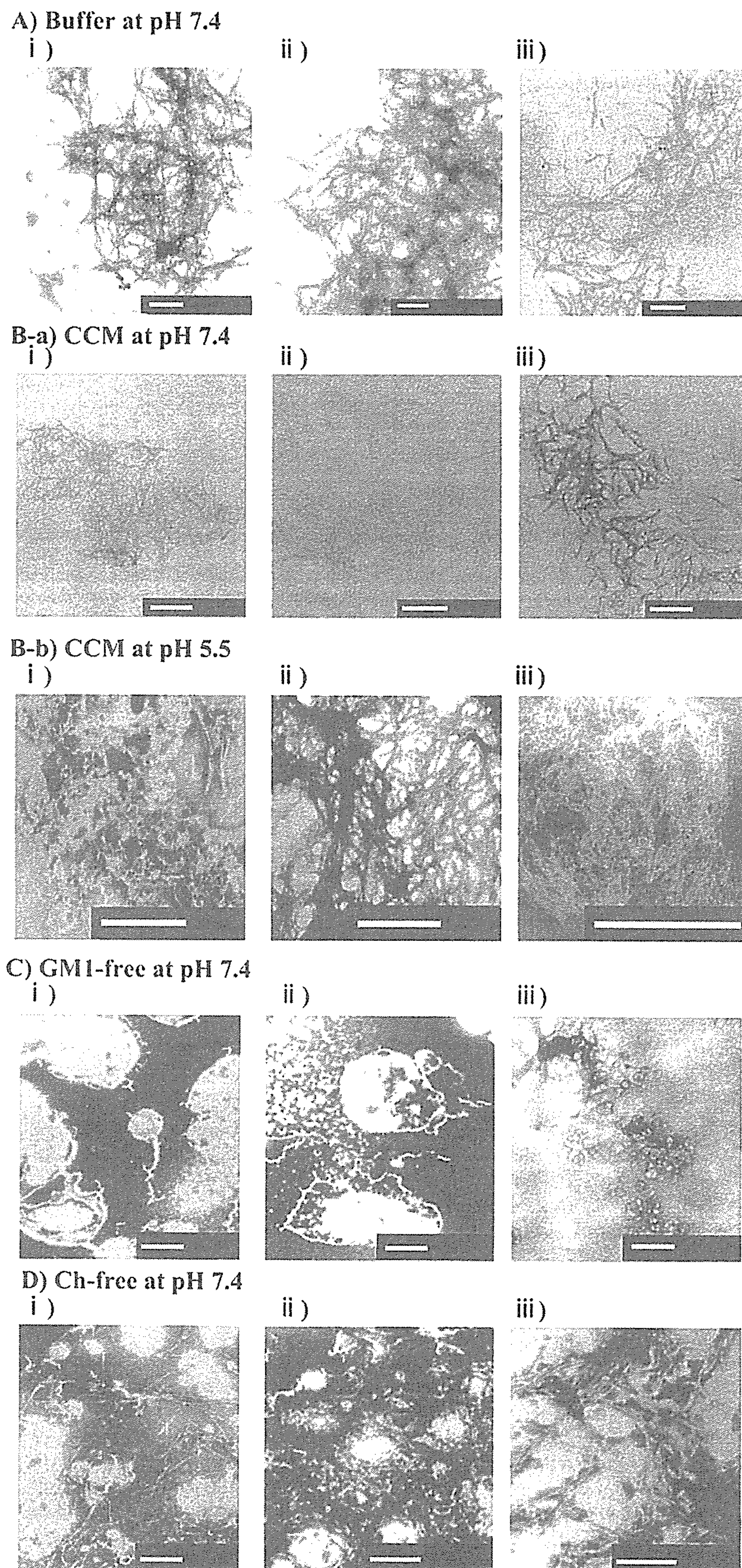

FIG. 4. **Electron micrographs in buffer solution (*A*), CCM at (*a*) pH 7.4 and at (*b*) pH 5.5 (*B*), GM1-free (*C*), Ch-free (*D*), Ch-GM1-free (*E*) at (*a*) pH 7.4 and at (*b*) pH 5.5 over a period of 14 days after the incubation of Aβ-(1–40) and in CCM (*F*) and Ch-GM1-free liposomes (*G*) of Aβ-(1–40)/Aβ-(1–42)(10:1).** Images: 1 day (i), 3 days (ii), 10 days (iii) after liposomes were prepared from 50 μM peptide and 1 mM lipid solutions, respectively. Bars are 500 nm.

eventually thicker and longer fibers. In Ch-GM1-free membranes at pH 7.4 and 5.5 (Fig. 4, *E-a* and *-b*) fibrils were not observed, liposomes of various shapes and sizes were present, and with time an increase in the number of smaller vesicles occurred.

Images of Aβ-(1–40)/Aβ-(1–42) (10:1) in CCM (Fig. 4*F*) were very similar to those for Aβ-(1–40) (Fig. 4*B*), showing indistinct fibril structures after 1 day and extensive well defined ones after 10 days (Fig. 4*F*). Similar images (not shown) were observed for GM1-free membranes. Interestingly, in Ch-GM1-