Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 241-248

HIGH MOLECULAR WEIGHT ALZHEIMER'S DISEASE AMYLOID PEPTIDE IMMUNOREACTIVITY IN HUMAN SERUM AND CSF IS AN IMMUNOGLOBULIN G

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Received March 27, 1987

A radioimmunoassay (RIA) was developed to detect the 4200 Dalton amyloid (A_4) peptide or it's precursor (A_4P) in human serum or cerebrospinal fluid (CSF). A synthetic peptide containing the first 28 amino acids of the 43 amino acid A_4 peptide was covalently coupled to bovine thyroglobulin and a polyclonal antiserum in rabbits was prepared. This antiserum was specific for vascular amyloid and neuritic plaques in Alzheimer's disease brain as detected by immunoperoxidase. The synthetic peptide, which has a tyrosine at residue 10, was iodinated with chloramine T and [^{125}I]iodine and was purified to homogeneity by C_4 reverse phase high performance liquid chromatography (HPLC). Extraction of human serum over a C_{18} Sep Pak cartridge indicated immunoreactive A_4 peptide was not detectable in human serum. Conversely, high molecular weight A_4 peptide immunoreactivity was detectable in human serum, at a concentration of 8.9 ± 1.2 pmol-eq./ml, and in human CSF, at a concentration of 0.25 ± 0.01 pmol-eq./ml, giving a CSF/serum ratio of 3.2%. The immunoreactivity in human serum was nearly completely removed by affinity deletion of serum immunoglobulin G (IgG), but not by affinity removal of IgA or IgM. Serum immunoreactivity was decreased 90% in hypogammaglobulinemia, and was increased 83% in human cord serum. There was no statistical difference in serum A_4 immunoreactivity in Alzheimer's serum or CSF. Serum immunoreactivity in Down's syndrome was increased 50%. These studies indicate the high molecular weight A_4P immunoreactivity in human serum or CSF is an IgG. Whether the A_4 precursor in Alzheimer's disease is, in fact, an IgG, or whether there is an antibody in human serum and CSF that cross reacts with the A_4 precursor cannot be determined until the serum immunoreactivity is purified and structurally characterized. © 1987 Academic Press, Inc.

Alzheimer's disease is a neurodegenerative condition that affects approximately 2 million individuals in the United States and is a major leading cause of death in adults (1). Progress in the understanding of the pathogenesis of this condition has been rapid in the past three years. The principal neuropathologic lesions of Alzheimer's disease, i.e., neurofibrillary tangles, senile neuritic

0006-291X/87 $1.50

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plaques, and vascular amyloid, all bind the dye Congo Red and may be composed of the same amyloid peptide (2, 3). The initial purification and partial sequencing of a 4200 Dalton amyloid peptide (called β-peptide or A_4 peptide) was reported for amyloid isolated from extraparenchymal meningeal vessels in either Alzheimer's disease or Down's syndrome (4, 5) and, subsequently, from neuritic plaque amyloid cores (6), neurofibrillary tangles (7), and, recently, from intracortical microvessels of Alzheimer's disease brain (8). Based on the initial amino acid sequence (4, 5), oligonucleotide probes have been synthesized, cDNA libraries have been screened, and a portion of the precursor of the amyloid peptide has recently been cloned and sequenced by several laboratories (9-11). The gene encoding the putative amyloid peptide precursor (abbreviated A_4P) is on human chromosome 21 (9-11). This gene encodes a 3.5 kb transcript that is found in brain as well as a variety of peripheral tissues (9-11), and this transcript is increased in fetal brain and in brain of fetuses with Down's syndrome or trisomy 21 (10). The localization of the gene to chromosome 21 is of interest since virtually all subjects with Down's syndrome over the age of 40 develop Alzheimer's disease (5).

There are several important questions regarding the pathogenesis of Alzheimer's disease. First, does the A_4P protein arise primarily from brain or blood? If the precursor protein arises primarily from brain, then the CSF concentration of the A_4P protein may be higher than that found in blood. For example, the amyloid peptide in hereditary cerebral hemorrhage with amyloidosis (HCHWA) arises from the cerebral synthesis of the amyloid precursor, gamma trace, and the CSF concentration of this protein exceeds the plasma concentration (12). Second, if the A_4P protein does arise from blood, what kind of plasma protein is it? Is it an acute phase reactant protein, similar to the serum amyloid associated protein that causes amyloidosis involving peripheral tissues (13)? Third, does Alzheimer's disease or Down's syndrome-related Alzheimer's disease arise from the overproduction of the A_4P protein, causing the serum concentration to be elevated? Fourth, since animals such as polar bears, monkeys, or orangutans are known to develop vascular amyloid and neuritic plaques with age (14, 15), is the putative A_4P protein found in animal serum? In an attempt to answer these questions, an RIA for detecting the A_4 peptide or the A_4P protein in human serum or CSF was developed.

METHODS

Materials - A 28 amino acid peptide corresponding to the first 28 amino acids of the A_4 peptide (4-7) was synthesized and sequenced in

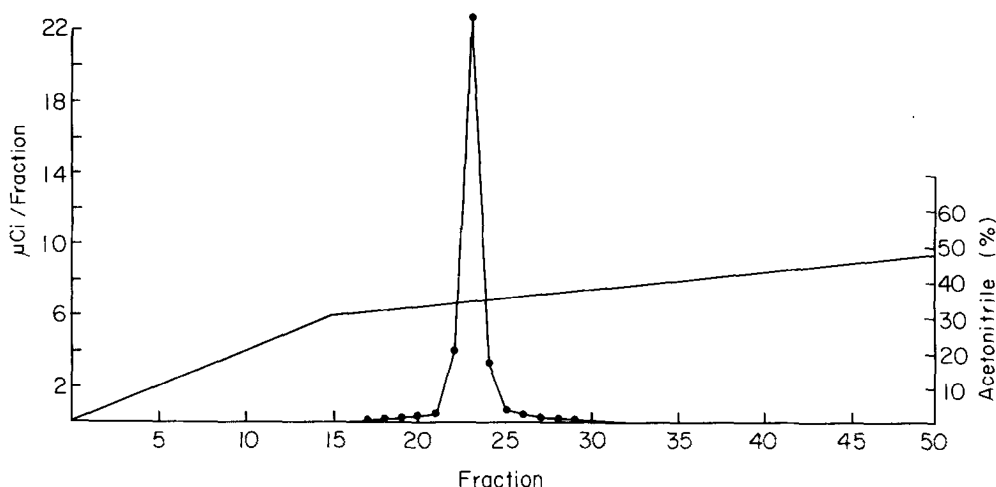

Figure 1. Elution of [^{125}I]synthetic peptide from a C_4 reverse phase HPLC column with an acetonitrile gradient.

the UCLA Department of Biochemistry. [^{125}I]iodine was obtained from New England Nuclear Corporation (Boston, MA). Pansorbin was purchased from Calbiochem (La Jolla, CA). Human serum affinity deleted of IgG, IgA, or IgM was obtained from Cooper Biomedical (Malvern, PA). Animal serum was obtained from the Los Angeles Zoo, with the exception of armadillo serum, which was obtained from the Florida Institute of Technology (Melbourne, FL). All Alzheimer's disease patients met restrictive diagnostic guidelines developed by the NINCDS-ADRDA Workshop and fell into the "probable" diagnostic category (16).

Radioimmunoassay - The synthetic peptide was covalently coupled to bovine thyroglobulin using glutaraldehyde (17), and a polyclonal antiserum in rabbits was prepared. The specificity of this antiserum has been characterized previously using immunoperoxidase studies (18). The antiserum selectively illuminates vascular amyloid and neuritic plaque cores, but not neurofibrillary tangles in formalin-fixed, paraffin-embedded sections of Alzheimer's disease brain, but does not react with control brain. The antiserum also does not react with formalin-fixed, paraffin-embedded sections of postmortem human heart, kidney, lung, or spleen, but does illuminate the reticular epithelial cells of human thymus (18). The immunostaining of the vascular amyloid, the neuritic plaque amyloid, or the thymic reticular epithelium is abolished by absorption of the antiserum with the synthetic peptide (18). The synthetic peptide was iodinated with chloramine T and [^{125}I]iodine to a specific activity of ~ 150 μCi/μg. The iodinated synthetic peptide was purified by elution from a 4.6 x 250 mm 5 μ C_4 reverse phase column (Vydac, Hesperia, CA) with an acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) at 1 ml/min. The iodinated peptide eluted as a single peak at ~ 37% acetonitrile (Figure 1).

The RIA was performed by adding 100 μL of a 1:2,000 dilution of antiserum (final dilution = 1:6,000) in 0.05 M NaH_2PO_4 (PBW, pH = 7.4), 10 μL of [^{125}I]synthetic peptide (~ 15,000 cpm per tube), 100 μL of synthetic peptide standard in 0.05 M PBW (1% Triton X-100) or 10-100 μL of test serum or concentrated CSF, and the final volume was made 300 μL with the addition of 0.05 M PBW (1% Triton X-100). CSF was concentrated by evaporating 75-1,000 μL of CSF to dryness followed by resuspension in 100 μL PBW (1% Triton X-100). The RIA mixture was incubated at room temperature for 90 minutes followed by the addition

of 50 µL of Pansorbin (staphylococcal protein A), incubation for 30 minutes on ice, and centrifugation at 1,000 g for 20 minutes at 4°C. The supernatant was discarded and the pellet was counted for [^{125}I] to compute the total percent bound [^{125}I]. The percent bound for each sample was compared to the standard curve and the A_4P immunoreactivity was expressed as pmol/ml equivalent (eq.) to the pmol/ml for the synthetic standard.

Extraction of Serum - An 0.5 ml aliquot of serum was added to an activated C_{18} Sep Pak cartridge (Millipore, Bedford, MA) and, after washing with 5 ml of 5% acetonitrile in 0.1% TFA, the sample was eluted with 4 ml of 60% acetonitrile in 0.1% TFA followed by evaporation to dryness and resuspension in 0.5 ml of 0.05 M PBW (pH = 7.4, 2% Triton X-100). This extraction procedure resulted in a complete recovery of [^{125}I]synthetic 28 amino acid peptide.

RESULTS AND DISCUSSION

As shown in Figure 2, aliquots of control human serum ranging from 10-100 µL or aliquots of control human CSF ranging from 75-1,000 µL resulted in displacement of [^{125}I]peptide from the antiserum that was parallel to the synthetic peptide standard. The parallel displacement curves provided evidence that the immunoreactive substance

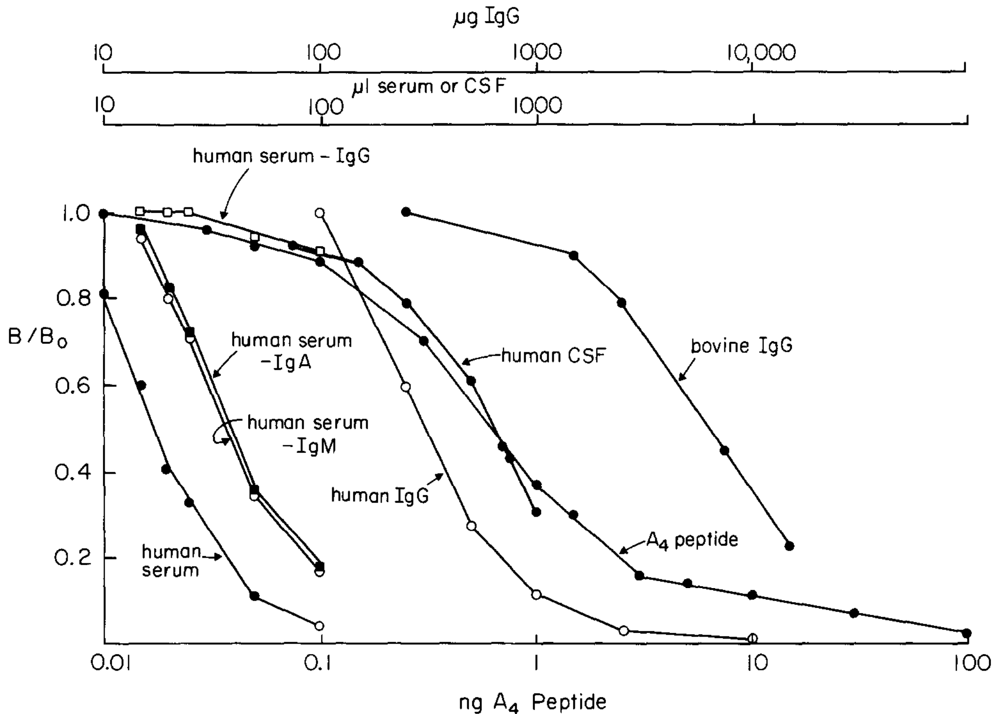

Figure 2. Displacement of [^{125}I]synthetic peptide from the rabbit antiserum by human serum, by human serum affinity deleted of IgG, IgA, or IgM, by a human IgG fraction, by human CSF, by synthetic A_4 peptide, or by a bovine IgG fraction. The data are expressed as B/B°, where B = the [^{125}I] bound counts in the presence of the respective additive, and B° = the maximal [^{125}I] bound counts in the absence of any additive.

Table 1. Concentration of Alzheimer's Disease Amyloid Peptide Immunoreactivity in Human Serum and Cerebrospinal Fluid (CSF)

Group	CSF (n) (pmol-eq./ml)	Serum (n) (pmol-eq./ml)
Cord serum	-	13.0 ± 1.5 (11)**
Down's syndrome	-	10.7 ± 1.7 (12)*
Cirrhosis	-	10.6 ± 1.4 (12)*
Alzheimer's disease	0.25 ± 0.01 (17)	8.9 ± 1.2 (13)
Rheumatoid arthritis	-	8.8 ± 0.8 (4)
Normal	0.23 ± 0.01 (12)	7.1 ± 0.8 (10)
Pregnancy	-	6.3 ± 0.9 (10
Multiple sclerosis	0.073 ± 0.017 (6)**	5.3 ± 0.6 (4)*
Metastatic cancer	-	4.9 ± 1.4 (5)
Hypogammaglobulinemia	-	1.0 ± 0.2 (7)**

* $p < 0.05$ difference from normal
** $p < 0.005$ difference from normal
n = number of individuals per group

in human serum or CSF had an amino acid sequence highly homologous, if not identical, to either the A_4 peptide or it's putative precursor protein (A_4P). Extraction of human serum over a C_{18} Sep Pak cartridge using a protocol that quantitatively recovered the 28 amino acid synthetic peptide resulted in complete loss of detectable immunoreactivity from 1 ml of Alzheimer's disease serum. This suggested that the immunoreactive substance in serum or CSF was a high molecular weight protein that was highly homologous or identical to the A_4 peptide precursor, but was not the amyloid peptide, per se.

Various plasma proteins were screened to test for interference in the RIA and the following plasma proteins showed no signficant displacement of [^{125}I]peptide from the antiserum: human albumin (25 mg/ml), bovine thyroglobulin (10 µg/ml), porcine insulin (10 µg/ml), human prealbumin (600 µg/ml), and human transferrin (600 µg/ml). However, human IgG caused parallel displacement of the labeled synthetic peptide from the antiserum (Figure 2). Similarly, bovine IgG also caused parallel displacement of radioactive peptide from the antibody but was only about 5% as effective as the human IgG. Human serum that was affinity deleted of IgG, but not IgA or IgM, showed insignificant displacement of the synthetic peptide from the antiserum (Figure 2).

Nearly 90 different human sera and 35 CSF samples were screened in the RIA (Table 1). The normal serum concentration of A_4P immunoreactivity (7.1 pmol-eq./ml) was over thirty-fold greater than the normal CSF concentration (0.23 pmol-eq./ml), and the average CSF to serum ratio was 3.2%. There was no significant difference in the serum or CSF concentration of A_4P immunoreactivity in Alzheimer's dis-

ease as compared to controls. The serum concentration of A_4P immunoreactivity was increased 83% in cord serum, although there was no significant difference in the concentration in pregnancy serum. The serum A_4P immunoreactivity was increased 49% in cirrhosis, and was decreased 25% in multiple sclerosis. The CSF concentration of A_4P immunoreactivity was also decreased in multiple sclerosis. The serum concentration was not elevated in inflammatory conditions such as rheumatory arthritis or metastatic cancer, which are known to have increases in serum acute phase reactants (19). The serum A_4P immunoreactivity was decreased 86% in hypogammaglobulinemia and was increased 51% in serum of subjects with Down's syndrome (Table 1).

Since aged animals develop amyloid angiopathy (14, 15), the sera of 17 different zoo or laboratory animals were screened for the presence of A_4P immunoreactivity. Ruminants such as the domestic or mountain goat, the sable antelope, the Dall sheep, or the Bezoar ibex, had serum concentrations less than 0.2 pmol-eq./ml. Other obligate herbivores, e.g., the Przwalski horse or the mountain zebra, also had nondetectable serum immunoreactivity. Conversely, the serum A_4P immunoreactivity in carnivores (striped hyena, jaguar, white tiger) and in opportunistic omnivores (e.g., gorilla, orangutan, squirrel monkey, or armadillo) was detectable and ranged from 2-15 pmol-eq./ml. The laboratory rat and African elephant had serum immunoreactivities between 0.5 and 1.0 pmol-eq./ml.

The present studies provide evidence that immunoreactive A_4 peptide is not detectable in Sep Pak extracted human serum. However, a high molecular weight immunoreactive substance is detectable in human serum and CSF, and human IgG causes displacement of the $[^{125}I]$synthetic peptide that is parallel to the standard curve (Figure 2). Additional evidence that the high molecular weight immunoreactivity in human serum is an IgG is given in Table 1, which shows a 90% reduction in serum immunoreactivity in hypogammaglobulinemia.

The proposal that the Alzheimer's disease A_4 peptide precursor is a IgG, however, is not consistent with the finding that the amyloid precursor protein is encoded on chromosome 21 (9-11), whereas the usual IgG heavy or light chain is encoded on human chromosomes 2, 14, and 22 (20). Moreover, the amino acid sequence of the A_4 peptide shows no homology with the amino acid sequence of known proteins (4-7, 9, 11). Therefore, one possible explanation of the present RIA data is that an antibody in human and animal serum, and in human CSF is highly homologous with the A_4 peptide and is cross reacting in the RIA. In this regard, Julliard et al (21) have shown previously that

an antiserum to β-endorphin cross reacts with a high molecular weight immunoreactive fragment in human placenta that is not a β-endorphin precursor, but is an IgG heavy chain. On the other hand, a finding that mitigates against the present RIA data representing a nonspecific antibody cross reactivity is the unexpected high concentration of immunoreactivity in human CSF. The ratio of immunoreactivity in CSF to serum is 3.2% (Table 1), and this ratio is thirteen-fold higher than the usual CSF to serum ratio for IgGs, which is 0.25% (22). The high CSF concentration of this IgG may arise from either selective transport of the antibody into human CSF from blood by specialized transport systems either at the blood-brain barrier or the blood-CSF barrier (23), or may reflect direct synthesis and neurosecretion of the immunoreactive species in brain. The discrimination between these two explanations of the present RIA data, i.e., antibody cross-reactivity versus identification of the A_4 peptide precursor of Alzheimer's disease as an IgG, cannot be made until the immunoreactivity in human serum is purified and amino acid sequences of tryptic peptides are determined.

ACKNOWLEDGEMENT

This research was supported by a contract from the California Department of Health Services, by a grant from the John Douglas French Foundation for Alzheimer's Disease, and by a UCLA Biomedical Research Support grant. Dawn Brown skillfully prepared the manuscript. The authors are indebted to Dr. E. Richard Stiehm for providing the hypogammaglobulinemic serum, to Dr. Arvan Fluharty for the Down's syndrome serum, and to Dr. William T. Cefalu for the zoo animal serum.

REFERENCES

1. Terry, R.D. and Katzman, R. (1983) Ann. Neurol. 14:497-506.
2. Kidd, M., Allsop, D., and Landon, M. (1985) The Lancet 86:278.
3. Gajdusek, D.C. (1986) Neurobiol. Aging 7:453-454.
4. Glenner, G.G. and Wong, C.W. (1984) Biochem. Biophys. Res. Comm. 120:885-890.
5. Glenner, G.G. and Wong, C.W. (1984) Biochem. Biophys. Res. Comm. 122:1131-1135.
6. Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L., and Beyreuther, K. (1985) Proc. Natl. Acad. Sci., U.S.A. 3:58-68.
7. Masters, C.L., Multhaup, G., Simms, G., Pottgiesser, J., Martins, R.N., and Beyreuther, K. (1985) EMBO Journal 4:2757-2763.
8. Pardridge, W.M., Vinters, H.V., Jang, J., Eisenberg, J., Choi, T.B., Tourtellotte, W.W., Huebner, V., and Shively, J.E. (1987) J. Neurochem. (in press).

9. Goldgaber, D., Lerman, M.I., McBride, O.W., Saffiotti, U., and Gajdusek, D.C. (1987) Science 235:877-880.
10. Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, B.A.P., St. George-Hyslop, P., Van Keuren, M.L., Patterson, D., Pagan, S., Kurnit, D.M., and Neve, R.L. (1987) Science 235:880-884.
11. Kang, J., Lemaire, H-G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K-H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) Nature 325:733-736.
12. Ghiso, J., Jensson, O., and Frangione, B. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:2974-2978.
13. Janssen, S., van Rijswijk, M.H., Meijer, S., Ruinen, L., and van der Hem, G.K. (1986) In Amyloidosis (J. Marrink and M.H. van Rijswijk, eds.), pp. 61-72. M. Nijhoff, Boston.
14. Dayan, A.D. (1971) Brain 94:31-42.
15. Selkoe, D.J., Bell, D.S., Podlisny, M.B., Price, D.L., and Cork, L.C. (1987) Science 235:873-877.
16. McKhann, G., Drachman, D., Folstein, M., Katzman, R., and Price, D. (1984) Neurol. 34:939-945.
17. Reichlin, M., Schnure, J.J., and Vance, V.K. (1968) Proc. Soc. Exp. Biol. Med. 128:347-350.
18. Vinters, H.V., Pardridge, W.M., and Yang, J. (1987) Human Pathol. (in press).
19. Pardridge, W.M., Sakiyama, R., and Fierer, G. (1983) J. Clin. Invest. 71:900-908.
20. Darnell, J., Lodish, H., and Baltimore, D. (1986) In Molecular Cell Biology, p. 1102. Scientific American Books, Inc., New York.
21. Julliard, J.H., Shibasaki, T., Ling, N., and Guillemin, R. (1980) Science 208:183-185.
22. Walsh, M.J. and Tourtellotte, W.W. (1983) In Multiple Sclerosis (J.F. Hallpike, C.W.M. Adams, and W.W. Tourtellotte, eds.), pp. 275-358. Williams and Wilkins, Baltimore.
23. Pardridge, W.M. (1986) Endocrine Rev. 7:314-330.