DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Rockville MD 20857

Our Reference: BB-IND 5059

17 JUN 1993

Robert P. Friedland, M.D.
Clinical Director
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University Hospitals of Cleveland
2074 Abington Road
Cleveland, OH 44106

Dear Dr. Friedland:

Subsequent to our review of your **Investigational New Drug Application (IND)** for "Technetium-Tc-99m Conjugated Murine Monoclonal Antibody (10H3) to Beta Amyloid Protein," and as discussed during the telephone conversation of May 11, 1993, between you and Dr. Robert Kozak of this agency, your proposed study under this IND has been placed on clinical hold.

Your IND is on clinical hold because insufficient information has been submitted to allow FDA to assess the risks to the subjects in the proposed clinical investigation [21 CFR 312.42(b)(1)(iv)]. BB-MF 4986, submitted by NeoRx in support of your IND, is deficient in a number of areas involving manufacturing data documentation. We have sent NeoRx a letter requesting that additional information be submitted to their Master File. We also have the following comments and requests for further information:

PRODUCT AND MANUFACTURING INFORMATION

1. A complete description of the immunoreactivity assay along with controls and a description of the sensitivity of the assay is needed in order to evaluate the functional integrity of the Fab. Please provide all immunoreactivity data on Fab preparations before and after labelling. The technetium labelling of the Fab fragment is being done in the Department of Radiobiology at University Hospital. Will having the immunoreactivity assay performed at a distant site (by Dr. Marotta) be a problem for routine quality control testing of the labelled product? Please comment.

2. The master cell bank was characterized for adventitious agents. As part of the assessment for exogenously introduced contaminants, (A) an <u>in vitro</u> incubation of culture supernatant or cell line lysate should be performed with three separate indicator cell lines, and (B) an <u>in vivo</u> animal study should be performed in adult and suckling mice along with embryonated hens eggs in order to document absence of viruses other than retroviruses.

CLINICAL INFORMATION

3. We note the plan to study 15 subjects with mild disease and 15 subjects with more severe disease, and to compare severity of disease with intensity of the image and with the pattern of amyloid deposition. We also note that patients will be injected with a range of 2.5 mg to 10 mg of antibody, and a range of 15 to 40 mCi of Technetium. It is not clear how meaningful comparisons between patient groups can be made if the amounts of antibody and or label are so variable. An appropriate approach for the initial introduction of this antibody into humans is to select a homogenous population, such as those with more severe disease. These patients should be treated in cohorts, with each cohort receiving a higher dose of antibody (or radiolabel) than the preceding cohort. This will provide information on safety, on whether or not the antibody can image amyloid deposits in subjects with Alzheimer's disease, and may permit an assessment regarding dose response. Subsequent imaging studies could include subjects with less severe disease, and ultimately, subjects with other conditions which may also image with this antibody or which are included in the differential diagnosis of the target patient population.

<u>You may not initiate clinical trials under this IND</u> until your response to the above deficiencies has been received and reviewed by FDA, and you are informed that the response is satisfactory.

We have the following additional comments and questions:

PRODUCT AND MANUFACTURING INFORMATION

4. Please include a discussion as to the anticipated ease or difficultly in the labelled Fab fragment reaching sites of amyloid deposition. How will the blood-brain barrier influence uptake of labelled Fab?

5. Isolated monocytes were found to stain positively with the Fab fragment but this staining was thought to be non-specific. Since the antigen is available, could the specificity of the binding to isolated monocytes or monocyte lines be determined based on the ability of the synthesized A4 antigen to inhibit this binding?

CLINICAL INFORMATION

6. The protocol states that patients with more severe disease have an MMSE score < 17, while those with mild disease have a score of ≤ 18. Should this be corrected to state that mild disease is associated with a score of ≥ 18?

7. Please explain the rationale to compare smokers to non-smokers.

8. We recommend that, in addition to obtaining HAMA pre-study and at week 12, HAMA also be assayed at week 4. An evaluation only at week 12 may be too late to determine if HAMA has occurred. This has implications for repeat dosing and use of other murine monoclonal antibodies.

9. We recommend that the consent form discuss in more detail the theoretical risk of an acute hypersensitivity reaction with this agent.

10. We note that subjects will be imaged by SPECT at multiple times following the infusion of the antibody. Further clinical investigations with this radiolabeled antibody should focus on imaging subjects at those time(s) that yielded the best images in the Phase I studies.

11. The protocol states that subjects may be compensated up to $100.00 "if necessary to obtain volunteers." We are concerned about the use of financial inducements in this patient population. Please comment.

PHARMACOLOGY AND TOXICOLOGY INFORMATION

12. Information submitted to the IND does not adequately address the potential of cross-reactivity to non-target tissue. Please see the document "Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use (1987)." The following tissues should be included in examining the potential for cross-reactivity: lymph node, thymus, tonsil, bone marrow, blood cells, lung, liver, kidney, bladder, spleen, stomach, intestine, pancreas, parotid, thyroid, adrenal, pituitary, peripheral nerve, heart, striated muscle, ovary, testis, skin and eye.

If you have any questions, please contact Ms. Kay Schneider at (301) 295-9111.

Sincerely yours,

Janet Woodcock, M.D.
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July 7, 1993

Janet Woodcock, M.D., Acting Director
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Rockville, MD 20857

Re: Investigation new drug application (IND) "Technetium-Tc-99m conjugated murine monoclonal antibody (10H3) to ß amyloid protein" - DB-IND 5059

Dear Dr. Woodcock:

This letter is a preliminary response to your inquiry of June 17, 1993. We have addressed the clinical issues raised in your letter and we will be grateful for your review of this further information concerning our IND. Issues addressed are as follows:

1. (Question 4, from page 2) We do not expect the labeled Fab fragment to reach sites of amyloid deposition within the brain parenchyma, and we do not expect that the labeled Fab fragment will cross the blood brain barrier. Our expectation is that the labeled Fab will bind to antigen sites on the luminal surface of the cerebrovascular endothelium, which is not within the blood brain barrier. This prediction is based on extensive immunochemical and cytochemical studies in Alzheimer brain.

2. (Question 3, page 2) We will begin with patients with relatively more severe disease, as you suggest. Also, as you mentioned, we will start with lower doses of antibody in the range of 2.5mg. and will increase to 10mg. in later studies. This will provide information on safety and whether or not the antibody can image amyloid deposits in Alzheimer's disease. The comparison of patients with disease at different stages is a secondary question and will be addressed in the cohort studies you suggest with subjects with different stages of the disease being tested with the same dose of antibody. We will only study subjects with less severe disease following initial studies with patients who are relatively more severely effected.

2. (Question 6, page 2) The protocol is in error, as you point out, in regard to the MMSE cutoff for severe and mild disease. It should read that patients with more severe disease will have an MMSE score of less than 17, while those with mild disease will have a MMSE score of 18 or greater.

3. (Question 7, page 3) We will compare smokers to non-smokers because of the reported effect of smoking as diminishing the risk for Alzheimer's disease. (Van Duijn C, Hofman A, **British Medical Journal**, 1991; 302:1491-1494: Brenner DE et al., **Neurology**, 1993; 43:293-300). We believe that smokers with the disease will have relatively less amyloid deposition in brain vessels as demonstrated by binding of labeled Fab. This is a distinctly secondary hypotheses, and will not be tested until we examine the more primary issue of whether binding of the labeled Fab fragments in the brain of patients with Alzheimer's disease can be demonstrated.

4. (Question 8, page 3) We will add testing of HAMA at week four, as you request.

5. (Question 9, page 3) We have added more detail concerning the theoretical risk of an acute hypersensitivity reaction with the agent to the consent form.

6. (Question 10, page 3) We agree that further clinical investigation with the radio-labeled antibody fragment will focus on imaging subjects at those times that have been shown in our initial studies to yield the best images.

7. (Question 11, page 3) We are paying volunteers up to $100 because of the inconvenience involved in travel, expenses, and time. Some of our subjects must come from a considerable distance. The use of financial inducements is acceptable the University Hospitals of Cleveland Institutional Review Board.

Thank you for your attention to the IND. A further response concerning the issues raised in your letter concerning the master cell bank and immunoreactivity will be forthcoming.

Sincerely yours,

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Charles A. Marotta, M.D., Ph.D.
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August 5, 1993

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Rockville, MD 29857

Re: Investigation new drug application (IND) "Technetium-Tc-99m conjugated murine monoclonal antibody (10H3) to beta amyloid protein"- DB-IND 5059

Dear Dr. Woodcock:

This letter is a further response to your inquiry of June 17, 1993. We have addressed issues of the antibody assay and Fab characterization.

The procedures and results are as follows.

Preparation and characterization of the synthetic amyloid polypeptide

A 28 residue polypeptide homologous to ß/A4 was synthesized by the procedure of Merrifield. Amino acid analysis verified the structure. The amino acid sequence was reported by Masters and coworkers (1985) and is here referred to as ß/A4(1-28). ß/A4(1-28) is homologous, but non-identical, to the site occupied by amyloid precursor protein (APP) amino acids 597-624 (16-18). Peptide samples were stored as a dry powder at -20°C until used.

Preparation of Monoclonal Antibodies (Mabs)

Balb/c mice were injected subdermally with 1 mg each of antigen in Freund's complete adjuvant. After 3 weeks sera were positive at a dilution of 1/1,000 using the assays described. At 5 and 4 days prior to fusion 100 µg of antigen was injected both subcutaneously and intraperitoneally in phosphate buffered saline. Spleen cells were isolated and fused with plasmacytoma P3 NS1/1-4 Ag-1 cells (Majocha, et al, 1988).

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Supernatants were tested for antibody activity after 10-14 days using the assay procedures described below. Positive colonies were subcloned by limiting dilution and used in further experimentation.

Hybridomas were propagated as described in the procedures documented in the IND application using GMP conditions.

Antibody Purification

For studies on the purified antibody the latter was obtained from mouse ascites fluid in a single step using Protein G Sepharose Fast Flow (Pharmacia) following the manufacturers instructions.

Antibody Fragmentation

Fab fragments of 10H3 were obtained after enzymatic digestion of the whole antibody. Papain bound to agarose beads was obtained from Pierce and activated according to the manufacturer's instructions. 10H3 was exchanged into digestion buffer (0.02 M sodium phosphate, Ph 8.5, 0.003 M EDTA, 0.02 M L-cysteine, and adjusted to 5 mg/ml. Antibody was mixed with activated papain beads in a ratio of 1 mg of protein per 0.05 ml packed volume of beads. After 18 hr mixing at 37°C the mixture was centrifuged and the supernatant applied to a 5 ml column of Q Sepharose Fast Flow (Pharmacia) equilibrated with 0.1 M sodium phosphate, Ph 8.5. The flow-through volume containing pure 10H3 Fab was collected and concentrated to 20 mg/ml. The Fab fragment was pure as judged by SDS-PAGE and gel filtration HPLC.

Radiolabeling of 10H3 Fab with Technetium-99m (^{99m}Tc)

The procedrues of Fritzberg, et al, (1986) and Kasina, et al (1991) were used.

The Fab fragment was radiolabeled with generator-produced ^{99m}Tc using the diamide dimercaptide bifunctional chelating agent (Kasina, et al, 1991). Briefly, generator produced ^{99m}Tc pertechnetate was reduced by stannous ion and complexed gluconate ion. The reduced ^{99m}Tc gluconate was added to an acidified solution containing the diamide dimercaptide ligand. The ^{99m}Tc was

transchelated into the ligand during a 15 min incubation at 75°C. The ^{99m}Tc ligand solution was cooled to room temperature and adjusted to Ph 9.5 with bicarbonate buffer. A solution of antibody in phosphate-buffered saline was added to the ^{99m}Tc ligand solution and incubated at room temperature for 20 min. The ^{99m}Tc labeled antibody was then purified by anion exchange chromatography and diluted with normal saline prior to further use.

Dot Blot Assay: Quantitative/qualitative immunochemistry: prelabeled and postlabeled Fab

All antibodies were tested by the immunodot procedure that we developed and have used successfully in numerous studies (e.g., Majocha, et al, 1988). The detailed procedure is as follows:

Antibodies were tested for reactivity to antigen using the BioRad dot blot apparatus according to the manufacturer's directions. For initial screening, 1 mg of antigen was sonicated in 0.5 ml of 1% sodium dodecyl sulfate in H_20 and added to an equal volume of 2.5% Triton X-100, 0.3 M NaCl, 40 Mm Tris Hcl, Ph 7.4. Two μg beta AP was added to each well followed by 50 μl of 10% BSA. For Mab assays, 150 μl of culture supernatant or diluted purified fab fragment was added per well (see below for concentrations). After filtration TBS was used for intermediate washes between primary and secondary antibody additions and prior to addition of substrate. 50 μl of horseradish peroxidase-conjugated affinity purified, goat anti-mouse (Cappel), diluted 1/2,000 in 5% BSA, 0.5% Triton X-100, 0.15 M NaCl, 20 Mm Tris hydrochloride, Ph 7.4 was added to each well. The reaction product was visualized using diaminobenzidine, 0.5 mg/ml, imidazole, 1 mg/ml, and H_2O_2, 0.015%. Negative controls consisted of tissue culture media, omission of antigen, or addition of a monoclonal antibody supernatant specific for a protein other than antigen. These data were reported previously (Majocha, et al, 1992).

Fabs were labeled with ^{99m}Tc as described. After purification the Fabs were tested for retention of activity by the general dot blot

procedures. The concentration of pre-labeled and post-labeled Fab 10H3 was determined and then diluted to final concentrations of 0.2, 0.1 and 0.05 ug/ml. By means of the dot blot procedure it was shown that the pre-labeled and post-labeled Fab fragments detected as little as 2 μg of antigen after dilutions (Table I):

TABLE I: Comparison of Immunochemical Reactivity of 10H3 Fab Before and After Radiolabeling with ^{99m}Tc Using the Dot Blot Assay*

Fab (ug/ml)	10H3 Fab Before labeling	10H3 Fab After labeling
0.2	1.0	0.48
0.1	0.74	0.35
0.05	0.64	0.25

*Fab fragments were incubated overnight with antigen (2 ug/dot) followed by a 2 hour incubation with HRP-conjugated secondary antibody. Staining was visualized by diaminobenzidine. Dots were subjected to image analysis processing for quantitation.

We used the following immunohistological quantitation method to further characterized the Fab fragments. In this case the target antigen is Alzheimer brain senile plaque antigen.

Postmortem Brain Tissues

Postmortem brain material was obtained from the Brain Tissue Resource Center at McLean Hospital, Belmont, MA 02178. The brain used for evaluating 10H3 was a an 88 year old male with the clinical diagnosis of Familial AD. The diagnosis was confirmed upon postmortem examination which demonstrated the widespread occurrence of senile plaques and neurofibrillary tangles in all brain areas examined. In addition, congophylic angiopathy

was noted to involve intraparenchymal vessels as well as vessels within the subarachnoid space. The neurologically normal control brain used in the present study was that of a 65 year old male. Brain sections failed to stain with Thioflavin S.

Immunohistology

Formalin fixed human postmortem brain tissue was kept in 30% sucrose overnight and serially sectioned on a cryostat at 40 μm. After storage in glycerol/water (1:1)-1% sodium azide at -20° C, they were rinsed in Tris-buffered saline (TBST (0.9% NaCl, 20mM Tris, Ph 7.4), immersed in 1% H_2O_2 in TBS for 30 min and then rinsed in TBS. Each section was placed in the appropriately diluted primary antibody in immunocytochemistry primary buffer (IPB) (10% goat serum, 2% BSA in TBST [TBS + 0.5% Triton X-100]) overnight. After rinsing with TBST sections were treated with biotinylated goat anti-mouse IgG [Jackson ImmunoResearch Laboratories, diluted 1:200 in immunocytochemistry secondary buffer (ISB) (2% BSA in TBST)] for 2 hr and then rinsed with TBST. Sections were combined with Avidin DH: biotinylated horseradish peroxidase H complex (Vector) according to the manufacturer's directions, diluted with ISB, rinsed in TBST, developed for 2 min in a diaminobenzidine tetrahydrochloride solution, and mounted and coverslipped.

Quantitation of immunostained plaques

A quantitative assay for antibody specificity was carried out on sections of AD cortex. The numbers of immunostained plaques were counted using a scored grid inserted into the microscope eyepiece. Each grid was 0.25 mm per side; 319 adjacent grids were counted per tissue section for a total area of 19.94 mm^2. Since serial sections were used when comparing antibodies, data from a particular dilution series could be compared within that series with relative certainty that sections contained comparable numbers of plaques.

Immunochemical quantitation of Mabs: prelabeled and postlabeled 10H3

To assess retention of specificity for

amyloid accumulations in the AD brain, pre-labeled and post-labeled 10H3 Fab fragments were applied to AD cortex. Both before and after attachment of the diamide dimercaptide chelate of ^{99m}Tc the Fab exhibited strong affinity for amyloid deposits in blood vessels and parenchyma without a reduction in specificity, as indicated in Table II:

TABLE II: Comparison of Immunohistological Reactivity of 10H3 Fab Before and After Radiolabeling with ^{99m}Tc*

	Concentration (μg/ml)	Plaques per standard section of AD brain tissue
Unlabeled	1	224
Labeled	1	261
Unlabeled	5	586
Labeled	5	576

*The number of immunostained plaques determined by the standard assay method are indicated for 10H3 Fab fragments before and after labeling with ^{99m}Tc.

From our earliest studies on anti-amyloid Mabs we consistently documented that the antibodies are far more sensitive than traditional Thioflavin S staining for detection of senile plaques of the AD brain (Majocha, et al, 1988). We used this comparison to further characterize the 10H3 Fab before and after labeling with Tc.

In this study a section of AD brain tissue was immunostained with the 10H3 Mabs and compared for sensitivity to Thioflavin S. Quantitation is indicated by the numbers of plaques that could be visualized.

Thioflavin S Staining

Formalin-fixed sections were placed in a 0.1% solution of Thioflavin S (Sigma) in TBS

for 10 min. Excess stain was removed by placing the tissue sections in 70% ethanol for 1 to 2 min and then in water. Sections were coverslipped using a solution containing 20% polyvinyl alcohol (Sigma), 10% glycerol and 50 Mm Tris HCl, Ph 8.5.

Light Microscopy

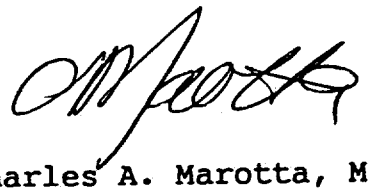
All photographs are taken with a Zeiss MC63 photographic system attached to a Zeiss standard microscope. Structures stained by Thioflavin S or fluorescent antibodies are visualized with an epifluorescent illuminator equipped with a fluorescein excitation and barrier filter set (Table III).

TABLE III: Comparison of Immunohistological Reactivity of 10H3 Fab Before and After Radiolabeling with ^{99m}Tc Compared to Thioflavin S Sensitivity.

	10H3 Fab detectable Plaques per standard section of AD brain tissue (adjacent sections)	Thioflavin S detectable plaques per standard section of AD brain tissue (adjacent sections)
Unlabeled (1 ug/ml)	224	173
Unlabeled (5 ug/ml)	586	
Labeled (1 ug/ml)	261	142
Labeled (5 ug/ml)	576	

This information presents the information that was requested on the 10H3 antibody Fab fragment. Please contact me if further elaboration is required.

Very truly yours,



Charles A. Marotta, M.D., Ph.D.

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