<table>
<thead>
<tr>
<th>タイトル</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroprotective Effect of D-Fructose-1,6-Bisphosphate against beta-Amyloid Induced Neurotoxicity in Rat Hippocampal Organotypic Slice Culture: Involvement of PLC and MEK/ERK Signaling Pathways</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>著者</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Song, Xiuzhen / Wu, Bin / Takata, Toshihiro / Wang, Xiaonan / Oizumi, Ximena Sayuri / Akisaki, Taichi / Yokono, Koichi / Sakurai, Takashi</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>掲載誌・巻号・ページ</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Kobe journal of the medical sciences,51(5/6):73-83</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>刊行日</th>
<th>Issue date</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006-01</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>資源タイプ</th>
<th>Resource Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Departmental Bulletin Paper / 紀要論文</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>版区分</th>
<th>Resource Version</th>
</tr>
</thead>
<tbody>
<tr>
<td>publisher</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>権利</th>
<th>Rights</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DOI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>URL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><a href="http://www.lib.kobe-u.ac.jp/handle_kernel/00422095">http://www.lib.kobe-u.ac.jp/handle_kernel/00422095</a></td>
<td></td>
</tr>
</tbody>
</table>

Create Date: 2017-12-22
Neuroprotective Effect of D-Fructose-1,6-Bisphosphate against β-Amyloid Induced Neurotoxicity in Rat Hippocampal Organotypic Slice Culture: Involvement of PLC and MEK/ERK Signaling Pathways

XIUYCHEN SONG 1, BIN WU 2, TOSHIHIRO TAKATA 1, XIAONAN WANG 1, XIMENA-SAYURI OIZUMI 1, TAICHI AKISAKI 1, KOICHI YOKONO 1, and TAKASHI SAKURAI 1

1 Division of Internal and Geriatric Medicine, Kobe University Graduate School of Medicine
2 Open Research Center of Lifestyle-Related Diseases, Mukogawa-Women’s University, Nishinomiya, Japan

Received 24 November 2005 /Accepted 6 January 2006

Key Words: Alzheimer’s disease; D-fructose-1,6-bisphosphate; Amyloid Beta-peptide; Adenosine triphosphate; Phospholipase C; Mitogen activated extracellular signal protein kinase; Extracellular signal activated protein kinase

D-fructose-1,6-bisphosphate (FBP) is an endogenous intermediate of glycolytic pathway which has potent neuroprotective effect against various neurotoxic insults. This study examined whether FBP could antagonize the neurotoxicity induced by amyloid β-peptide (Aβ) in rat hippocampal organotypic slice cultures, and the possible mechanism was also explored. Treatment with FBP (concentration ranges from 1.7 mM to 10 mM) significantly decreased the cell death in hippocampal slices in the presence of Aβ at 24h, 48h and 72h, and this neuroprotective effect of FBP against Aβ was not in a dose-dependent manner, FBP 3.5 mM has better neuroprotective effect than that of other FBP concentration groups. Treatment with FBP slightly but significantly increases the ATP levels in hippocampal slices in the presence of Aβ. However, the increment of ATP levels was similar among various FBP concentration groups. Neuroprotective effect of FBP 3.5 mM against Aβ induced neurotoxicity in hippocampal slices was attenuated by addition of phospholipase C (PLC) inhibitor, U73122, mitogen activated extracellular signal protein kinase (MEK) inhibitor, U0126, or extracellular signal activated protein kinase (ERK) inhibitor, PD98059 at 24h, 48h and 72h. However, co-treatment with these three kinds of inhibitors did not change the FBP’s effect on ATP levels. Our results suggested FBP has neuroprotective effect against Aβ induced neurotoxicity in hippocampal slice cultures, and FBP plays role not only as an alternative energy source, but also a modulator of PLC and MEK/ERK pathways to regulate the cellular response and survival.

Alzheimer’s disease (AD) is a progressive senile dementia characterized by deposition of Aβ in the form of senile plaques and the microtubule associated protein tau as paired helical filaments. Aβ is a 4 kDa peptide of 39-42 residues which has multi neurotoxic effects leading to the dysfunction and death of neurons (33). Both in vitro and in vivo studies have confirmed the crucial role of Aβ in the development of AD. Progressive neuronal loss in AD is considered to be a consequence of the neurotoxic properties of Aβ (14). From this point of view, understanding the mechanism of Aβ neurotoxicity could provide potential therapeutic targets for AD treatment.

Alzheimer’s disease (AD) is a progressive senile dementia characterized by deposition of Aβ in the form of senile plaques and the microtubule associated protein tau as paired helical filaments. Aβ is a 4 kDa peptide of 39-42 residues which has multi neurotoxic effects leading to the dysfunction and death of neurons (33). Both in vitro and in vivo studies have confirmed the crucial role of Aβ in the development of AD. Progressive neuronal loss in AD is considered to be a consequence of the neurotoxic properties of Aβ (14). From this point of view, understanding the mechanism of Aβ neurotoxicity could provide potential therapeutic targets for AD treatment.

Phone: +81-798-4590-38  Fax: +81-798-4590-38  E-mail: Wubin@mwu.mukogawa-u.ac.jp
view, preventing the Aβ induced neurotoxicity is of great importance for the development of potent therapeutic strategies.

D-fructose-1,6-bisphosphate (FBP), an endogenous intermediate of glycolytic pathway, can protect organ system from lethal injury accompanying ischemia or shock (21). There is some other evidence that FBP attenuates brain damage induced by hypoxia-ischemia (15), insulin induced hypoglycemia (7) and cardiogenic shock (34). FBP is also reported to provide protection of neurons against stimulated ischemia in hippocampal slices (19). The mechanisms by which FBP protects the brain neurons are not well understood. Possible mechanisms of protection include anaerobic metabolism of FBP to yield ATP (9) or its ability to reduce ATP loss (11), calcium chelation (13) and modulation of second messenger system.

Up to date, whether the neurotoxicity of Aβ in hippocampus could be antagonized by FBP is not well documented. We conducted this study to examine if FBP has the neuroprotective effect against Aβ induced toxicity using organic hippocampal slices. Furthermore, to determine whether FBP serves as an alternative energy sources to preserve neuronal survival, we examined the effects of exogenous FBP on ATP levels in organic hippocampal slices during Aβ neurotoxication. Recently many studies also indicate that FBP exerts its neuroprotective effect by modulating intracellular signaling pathways. A few intracellular signaling pathways, such as PLC and MEK/ERK (6) are reported to be associated with the neuroprotective effects of FBP, and thus we investigated here if these pathways are involved in the effects of FBP on the Aβ induced neurotoxicity.

MATERIALS AND METHODS

The experiments were conducted according to the guidelines for animal experimentation at the Kobe University School of Medicine and conform to relevant National Institution of Health guidelines.

Preparation of organotypic hippocampal slices

Hippocampal slices were made from the septal half of the hippocampus using a standard method (28). Briefly, 9-11 days Wistar rats (Hartley, SLC, Japan) were anesthetized with 98% Diethyl Ether and decapitated. The hippocampi were rapidly dissected at 4-6°C and cut into 450μm slices using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co.Ltd, UK). Slices were then transferred onto 30-μm diameter-pored membrane (Millicell-CM, Millipore, Bedford, MA, USA), and put into a six-well microplate (Costar Corning Inc, NY, USA) with 1ml slice culture media per well. The culture media contained 50% Eagles minimal essential medium (MEM) (Gibco, CA, USA), 25% Hanks’ Balanced Salt Solution (HBSS) (Gibco, CA, USA), 25% heat inactivated horse serum (Gibco, CA, USA) containing 1% penicillin/streptomycin. Slices were kept in culture for 14 days before study and the six-well micropaltes were stored at 37°C in a 95% humidified atmosphere with 5% CO2 incubator (Sanyo, Tokyo, Japan).

Treatment of hippocampal slices

Slices in six-well micropaltes at day 14 were washed, and the basic medium was replaced with various agents for the treatment. The basic medium contained 90mM NaCl, 4mM KCl, 0.1mM MgCl2, 0.1mM KH2PO4, 0.5 mM MgSO4, 0.1 mM Na2HPO4, 0.5 mM NaH2PO4, 14 mM NaHCO3, 1.2 mM CaCl2, 10 mM glucose, about 2 mM essential and non-essential amino acids, 0.02 mM vitamins. To establish the Aβ induced neurotoxicity, slices were treated with three kinds of Aβ peptides (Aβ25-35, Aβ1-40, and Aβ1-42) in various concentrations. Aβ25-35, Aβ1-40, and Aβ1-42 (Peptide Institute Inc. Japan, Osaka) were dissolved in sterilized distill water. To assure full contact between Aβ and the culture,
treatment media was applied from underneath the insert onto the culture by pipetting at first 4h. Various concentrations of FBP (Sigma, St. Louis, MO, USA) were added to the culture with or without Aβ25-35 for determining the FBP’s effect against the Aβ induced neurotoxicity. To determine whether a signaling pathway is involved in the neuroprotective effect of FBP, a few signaling pathway-specific inhibitors were used, including a phospholipase-C (PLC) inhibitor, U73122 (Wako, Osaka, Japan), a mitogen activated extracellular signal protein kinase (MEK1/2) inhibitor, U0126 (Wako, Osaka, Japan), an extracellular signal activated protein kinase (ERK) inhibitor, PD98059 (Wako, Osaka, Japan), and a protein kinaseC (PKC) inhibitor, chelerythrine (Calbiochem Merck, Tokyo, Japan). Each pathway-specific inhibitors (10 μM) was added into the slice culture with or without various concentrations of FBP and Aβ25-35.

Assessment of cell death in hippocampal slices

Propidium iodide (PI) method was applied for the assessment of neuron death in hippocampal slices at 24h, 48h, and 72h after each treatment in the CA1 region of the hippocampus. To label the nuclei of dead neurons, 4.6μg /ml PI (Sigma, Louis.St, Mo, USA) was added to the wells of the culture microplates for 15 min. PI is a polar compound which only enters cells with damaged cell membranes. Inside the cells it binds to nucleic acids and becomes brightly red fluorescent. The dye is basically non-toxic to neurons and has been used as an indicator of neuronal integrity and cell viability (20). Thus the intensity of fluoresce is parallel to the cell death. After 15 min, digital imagines of PI fluorescence were obtained with an inverted fluorescence microscope (4× objective) equipped with a digital camera(Olympus IX70, Tokyo, Japan). After the final image, all the neurons were killed by adding 10 μM N-Methyl-D-Aspartic Acid (NMDA) and the final PI fluorescence intensity was adjusted equivalent to 100% cell death. The mean intensity (green values) of the PI fluorescence were measured using an image program MacScope (Ver 2.6.1, Mitani Inc, Osaka, Japan).

Measurement of ATP levels

Hippocampal slices were dissected under a microscope at 48h after each treatment. Four slices were immediately homogenized in 0.5 N perchloric acid with 1 mM thylene-diaminetetra acetic acid and centrifuged for 15min at 2000rpm. The supernatant was neutralized with 2M KHCO3, recentrifuged and stored at –30℃ until assay of ATP. ATP was quantitated enzymatically and fluorometrically by measuring the production of nicotinamide adenine dinucleotide phosphate hydride (29). Protein content of the slices was determined by the method of Lowry and Passonneau (25).

Statistical analysis

Date was expressed as mean ± standard error of the mean (s.e.m) from three independent experiments. Statistical significance was established by ANOVA followed by post-hoc test using SPSS (Ver 12.0, SPSS. Inc., Chicago, USA) software. P<0.05 was considered to be statistically significant.

RESULTS

Neurotoxicity of Aβ

Three different kinds of Aβ fragments, Aβ25-35, Aβ1-40, and Aβ1-42, were applied to establish the neurotoxicity of Aβ. Cell death was evaluated at 48h after various concentrations of three Aβ fragments administration. Aβ1-40, and Aβ1-42 caused up to 40%-70% cell death at concentrations ranging from 0.5 μM to 50 μM. Aβ25-35 (50μM) induced similar toxicity comparable to Aβ1-40, and Aβ1-42 at 25μM (data not shown). Since Aβ25-35 and full length Aβ1-42 cause neuron death by similar mechanisms (22), Aβ25-35 50μM was used in all subsequent experiments.
Neuroprotective effect of FBP in hippocampal slices

Various concentration of FBP (0 mM, 1.7 mM, 3.5 mM, 7 mM, 10 mM) were added to the media. Compared with control group (FBP 0 mM), the addition of FBP significantly reduced the cell death in hippocampal slices at 24h, 48h and 72h after treatment (shown in FIG. 1). Interestingly, this neuroprotective effect of FBP was not in a dose-dependent manner. Compared with other FBP concentration groups, FBP 3.5 mM has better neuroprotective effect than those of other FBP groups (FBP 3.5mM group vs other FBP concentration groups, all the $P<0.01$).

Neuroprotective effect of FBP against Aβ induced neurotoxicity in hippocampal slices

As shown in FIG.2, treatment with FBP significantly decreased Aβ induced cell death in hippocampal slices at 24h, 48h and 72h (All the FBP concentration groups compare with control group, $P<0.01$). Similarly, this neuroprotective effect of FBP against Aβ was not in a dose-dependent manner. FBP 3.5mM group has better neuroprotective effect than that of other FBP concentration groups(FBP 3.5mM+Aβ group vs other FBP concentration groups, all the $P<0.01$).

Neuroprotective effect of FBP against Aβ induced neurotoxicity was attenuated by PLC, MEK or ERK inhibitors

Some other studies suggested that the neuroprotective action of FBP against hypoxia was dependent on PLC, MEK/ERK pathways, and this was also found to be the case with the hippocampal slices when exposure to Aβ induced neurotoxicity. Protective effect of FBP 3.5 mM against Aβ induced neurotoxicity in hippocampal slices was abolished by PLC inhibitor, U73122, MEK inhibitor, U0126, and ERK inhibitor, PD98059 at 24h, 48h and 72h. However, administration of chelerythrine, a protein kinase C inhibitor, did not modulate the neuroprotection of FBP against Aβ induced neurotoxicity in hippocampal slices (FIG. 3).
FIG. 2. Neuroprotective effects of FBP against Aβ induced neurotoxicity on cultural hippocampal slices

![Graph showing cell death % of control over time for different FBP concentrations against Aβ]

**FIG. 2.** Neuroprotective effects of FBP against Aβ induced neurotoxicity on cultural hippocampal slices. Treatment with FBP significantly decreased Aβ induced cell death in hippocampal slices at 24h, 48h and 72h. FBP 3.5mM group has better neuroprotective effect than those of other FBP concentration groups (each n=39). ♦ : compare with control group (FBP 0 mM, n=36), P<0.01, ♦♦ : compare with FBP 3.5 mM group, P<0.01.

FIG. 3. PLC inhibitor, MEK inhibitor, and ERK inhibitor attenuated the neuroprotective effect of FBP 3.5mM against Aβ induced neurotoxicity in hippocampal slices

![Graph showing cell death % of control over time for different inhibitors against FBP and Aβ]

**FIG. 3.** Co-treatment with PLC inhibitor U73122 (n=45), MEK inhibitor U0126 (n=39), and ERK inhibitor PD98059 (n=48) attenuated the neuroprotective effect of FBP 3.5mM against Aβ induced neurotoxicity in hippocampal slices at 24h, 48h and 72h. Administration of chelerythrine, a protein kinase C inhibitor (n=36), did not modulate the neuroprotection of FBP 3.5 mM against Aβ induced neurotoxicity. ♦ : compare with control group (FBP 3.5mM + Aβ, n=48), P<0.01.
FIG. 4. Effects of FBP on the ATP levels of hippocampal slices in the absence of Aβ

Compared with control group (FBP 0 mM, n=48), FBP groups (concentration ranging from 1.7 mM to 10 mM, each n=36) had significant elevated ATP levels in the absence of Aβ in hippocampal slices at 24h and 48h. However, the ATP levels were not significantly different among these FBP groups. *: Compare with control group (FBP 0mM), P<0.001.

FIG. 5. Effects of FBP on the ATP levels of hippocampal slices in the presence of Aβ

The ATP levels were preserved at each concentration of FBP. However, the difference of the ATP levels among these various FBP concentration groups( each n=36) did not reach to significance. *:compare with FBP 0 +Aβ group(n=36), all the P<0.001.
Effects of FBP on the ATP levels of hippocampal slices in the presence or absence of Aβ

To test the hypothesis whether the neuroprotective action of FBP against Aβ induced neurotoxicity was due to its role as an alternative energy source, we examined the effect of various concentrations of FBP on the ATP levels in hippocampal slices in the presence or absence of Aβ. Compared with control group (FBP 0 mM), FBP groups (concentration ranging from 1.7 mM to 10 mM) had significant elevated ATP levels in hippocampal slices at 24h and 48h in the absence of Aβ (all the P<0.001). The ATP levels were not significantly different among these FBP groups (FIG.4). With the presence of Aβ, the results were similar with those without Aβ, and the ATP levels were preserved at each concentration of FBP (compare with FBP 0 mM+Aβ group, all the P<0.001). However, the difference of the ATP levels among these various FBP concentration groups did not reach to significance (FIG. 5).

Effects of PLC, MEK, ERK or PKC inhibitors on the ATP levels in hippocampal slices in the presence of FBP and Aβ

To investigate whether energy metabolism is involved in the neuroprotective action of FBP against Aβ toxicity through specific signaling pathways, ATP levels were examined when co-treated with specific inhibitors. Compared with control group (FBP 3.5 mM+ Aβ), the addition of PLC inhibitor, MEK inhibitor, ERK inhibitor or PKC inhibitor did not cause significant difference in the ATP levels in hippocampal slices at 24h and 48h (FIG.6).

**FIG. 6. Effects of PLC, MEK, ERK and PKC inhibitors on the ATP levels in hippocampal slices in the presence of FBP and Aβ**

![Graph showing ATP levels in hippocampal slices](image_url)
DISCUSSION

We have shown that exogenous FBP reduced Aβ induced cell toxicity in rat hippocampal slices. This neuroprotective effect of FBP might be the result of additional supply of ATP in the hippocampal slices. However, the neuroprotective action of FBP against Aβ induced neurotoxicity was not dose-dependent, and the ability of FBP to produce or preserve ATP was not dose-dependent, either. The results therefore suggest that protective action of FBP against Aβ induced neurotoxicity in hippocampal slices was due to, at least in part, other than its role as an alternative energy substrate to yield additional ATP. Furthermore, co-treatment of specific signaling pathways inhibitors with FBP and Aβ reduced the cell viability without altering ATP levels, suggesting that protective action of FBP against Aβ induced neurotoxicity was not only due to its role as an alternative energy source, but also a modulator for neuroprotective signaling pathways.

FBP has been shown to attenuate tissue damage resulted from myocardial or kidney ischemia (7, 4). Many studies demonstrated that FBP has neuroprotective effect in central nervous system against hypoxia/ischemia (30, 31). Here we showed that FBP attenuated the neuron death induced by Aβ in hippocampal slices in a non dose-dependent manner, and also the ability of FBP to preserve the ATP levels appears not to be related to its concentrations. Because of its role as an intermediate products in glycolysis, it has been widely assumed that the protective effects of exogenous FBP results from its serving as an additional substrate for glycolysis (12). But our findings didn’t seem to consist with this hypothesis since the increasing of FBP could not lead to the ATP elevation in a dose-dependent manner in hippocampal slices during Aβ exposure. To our knowledge, there are no specific transporters for FBP in the central nervous system. FBP is a highly negative charged molecular which is not easy to cross the hippocampal cellular membranes. Considering the fact that FBP was not taken up by red blood cell (26) or myocardial tissue (8), it was hypothesized the FBP would first have to undergo hydrolysis to fructose in order to be utilized. However some other experiments have demonstrated that, unlike FBP, addition of fructose or fructose-6-phosphate did not have neuroprotective effects (10). It seems that relatively small amounts of exogenous FBP could be metabolized by hippocampal slices.

While it seems only small amount of FBP could be uptaken by hippocampal culture cells, they are probably insufficient to explain its role as an energy substrate to maintain ATP level in hippocampal slice cultures. However, we could not exclude the possibility that this level of FBP might be sufficient to regulate energy metabolism and to modulate intracellular second messenger system. Some investigators illustrated that exogenous FBP has biphasic effects on the neuronal cellular metabolism. On one hand, FBP promotes glucose metabolism in astrocytes via pentose phosphate pathway (16). PPP is quite active in the CNS (35, 17) and stimulation of PPP may lead to the increasing production of NADPH, synthesis of fatty acids, triglycerides, and phospholipids, then reduces oxygen radical injury of neural cell by regulating glutamine peroxidase (18, 32). On the other hand, exogenous FBP may reduce the uptake of glucose from extracellular environment (16), and, moreover, it could inhibit the activity of phosphorfructokinase (PFK)-the key enzyme of glycolysis, therefore reduce the production of lactate and the activity of TCA cycle (16). Our findings that neither higher nor lower levels of FBP cause better neuroprotective effects could be partially explained by this dual effects of FBP on the metabolism of neural cells.

Besides its role for serving as a metabolism regulator in the neuroprotective effects of FBP, the possible involvement of FBP in several intracellular signaling pathways should be taken into account. Recent studies have shown that neuroprotective qualities of FBP on
hypoxia/ischemia-induced toxicity in hippocampal slices are dependent on PLC (5) and MEK/ERK pathways (6). In consistent with these studies, our observations also imply that PLC and ERK/MEK pathways are involved in the neuroprotective effects of FBP against Aβ induced neurotoxicity in hippocampal slices. Co-administration of PLC or ERK/MEK pathway inhibitors attenuate the neuroprotective action of FBP, but without affecting the ATP levels in hippocampal slices. Intracellular signaling pathways play crucial roles in regulating the cellular response and survival following insults by neurotoxins such as Aβ. Since little of exogenous FBP could enter neurons to serve as a signal, it was hypothesized that FBP might initiate its neuroprotective signaling at or near the cell surface. On the cell surface, FBP stimulate lipolysis (3) through PLC pathway and increase the production of diacylglycerol and inositol triphosphate, leading to the elevation of intracellular Ca\(^{2+}\). The consequence of this event is the activation of MEK/ERK signaling pathway and the expression of neuroprotective genes. Another important intracellular signaling system is PKC, a family of 12 serine/threonine kinase (23). Since PKC has been found to modulate cell viability resulting in the protection of various neuronal cells (27), we also investigated here if PKC pathway was involved in the neuroprotection of FBP. However, our data indicate that co-treatment of PKC inhibitor did not make significant alternations of both cell death and ATP levels in hippocampal slices in the presence of FBP and Aβ, suggesting that PKC signaling pathway may not be involved in the neuroprotective effects of FBP against Aβ induced neurotoxicity in hippocampal slices.

In the present study, we first report that FBP has neuroprotective effects against Aβ induced neurotoxicity in hippocampal slices. The preservation of ATP and the involvement of PLC and MEK/ERK signaling pathways could explain FBP’s role as a modulator for both energy metabolism and intracellular signaling pathways. Even more, some recent studies have revealed that FBP has immunomodulatory (2) and anti-inflammatory properties (1,24) in modulating cellular function. The mechanism of FBP’s neuroprotective effects seems to be multifactorial, and extensive studies are required to reveal its complex roles as a neuroprotectant.

ACKNOWLEDGEMENTS

This work was supported by a Research Grant from the Novartis Foundation for Gerontological Research and a Grant-in-Aid for Scientific Research (17500473) from Japan Society for the Promotion of Science. We deeply appreciate Dr. Sakaguchi, T., and Dr. Yang., B for technical assistance.

REFERENCES


