THE HEMATOPOIETIC STEM CELL THERAPY
FOR SPACE-CAUSED DISORDERS

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ABSTRACT

It is well documented that prolonged exposure to space environments causes severe/invasive disorders in astronauts. These include hematological/cardiac abnormalities, bone and muscle losses, immunodeficiency, neurological disorders and cancer. Exploiting extraordinary plasticity of HSCs, which can differentiate into all types of blood cells, as well as transdifferentiate into various tissues, we have hypothesized that the hematopoietic stem cell-based therapy, herein called as the hematopoietic stem cell therapy (HSCT), could mitigate some of the disorders mentioned above, so as to maintain astronauts homeostasis in space. If this were achievable, the HSCT can promote human exploration of deep space. Using animal models of disorders (β-thalassemia and hindlimb suspension unloading model), we studied the HSCT for space anemia, muscle loss, and immunodeficiency. To develop the HSCT protocol for space anemia, the HSC transplantation procedure was applied to a mouse model of β-thalassemia, yielding successful engraftment of HSCs and alleviation of anemia. To investigate the HSCT for muscle loss, donor HSCs were genetically marked either by transfecting the β-galactosidase-containing plasmid, pCMV.SPORT-β-gal or by preparing from β-galactosidase transgenic mice. The transdifferentiation of HSCs to muscle is traced by the reporter gene expression in the hindlimb suspended mice. The X-gal staining procedure and histochemical analysis indicated the contribution of HSCs to muscle. We have also initiated experiments using the SDS-PAGE, aiming at determining the frequency with which the HSCs should be administered. In the future, the HSCT will be applied to long-term bed rest patients to prevent their muscle loss. This study will lead to prevention of astronauts’ muscle loss in space. To investigate the HSCT for immunodeficiency, the ability of hindlimb suspended mice to eliminate bacterial infection was studied. Escherichia coli were transformed with a plasmid, pCMV.SPORT-β-gal, which were then used as the gene-marked bacteria to infect control and the hindlimb suspended mice. Results by the X-gal wholemount staining procedure indicate that the hindlimb suspension unloading indeed cause the immunodeficiency and the HSCT could help eliminate the reporter gene-marked E. coli. To quantitate this data, we have established protocols of determining colony forming units of bacteria in host blood. Preliminary results are concurrent with that observed in the wholemount X-gal staining. As a future project, exploiting the possibility of HSCs to augment host immunity, we have initiated investigation into the HSCT for cancer.

DESIGN PROBLEM

Our hypothesis is to maintain astronauts' homeostasis in space using hematopoietic stem cell therapy, so as to enable them to "go anywhere at any time".

INTRODUCTION

Several reports indicate that astronauts develop hematological abnormalities, including space flight anemia, abnormal red cell morphology and structure, thrombocytopenia, 5-20% reduction in red blood cell mass, decreased hemoglobin concentration and hematocrit, and lowered serum erythropoietin levels (1-5). It is likely that stem cell self-renewal is also inhibited in space, reducing the total number of totipotent stem cells in bone marrow. This abnormal hematopoiesis in 0/µ G could adversely affect the astronauts' homeostasis. One avenue to overcome the abnormal hematopoiesis might be to supply normal hematopoietic stem cells (HSCs) periodically to astronauts. The newly supplied HSCs could differentiate to normal blood cells, thus, maintaining the healthy hematological status of the astronaut for
some duration. This process could be repeated and made as routine throughout the long-duration space mission so as to maintain this equilibrium state. This is the essence of our proposed hematopoietic stem cell therapy (HSCT) in space; namely, the objective is to prevent the occurrence of these disorders: Preventive Medicine. And thus, the HSCT could be applied to various disorders in space, including space anemia, muscle and bone losses, immunodeficiency and cancer as discussed below. It must be emphasized that the HSCs should be prepared on the ground from individual astronaut’s blood preflight and kept frozen. The HSCs should be designated to each astronaut and should not be intermixed. During the flight the HSCs would be thawed and grown and expanded. Following the culture, a half of the HSCs would be used for transplantation and the other half frozen for the next culture. In this way, the individual HSCs would be maintained as a normal stock during the long-term space flight. In addition, the transplanted HSCs should be astronaut’s own, i.e. autologous transplantation, avoiding the danger of graft-versus-host disease (GVHD). Hence, no myeloablation by toxic chemicals, such as cyclophosphamide and busulfan, would be necessary. This project also necessitates optimizing protocols for frequency with which HSCs should be administered. This can be determined by analyzing histochemically the muscle fibers of HLS mice and determining how often HSCs should be administered to prevent any change in muscle fibers.

Growth of Hematopoietic Stem Cells in Space

The HSCT in space will necessitate establishing an optimal condition for growing normal HSCs in 0/μ gravity from the frozen state. The growth and expansion of HSCs in space could be achieved by the use of NASA Rotating Wall Vessel (RWV) system.

HSCT for Space Anemia: Transplantability of HSCs

Space anemia is the anemia experienced by astronauts when they return to earth from μ gravity conditions. Studies on hematopoiesis in space using human hematopoietic stem cells (CD 34+cells) in culture indicated that both proliferation and differentiation of blood cells were severely inhibited (by more than 50%) by 0/μ G, erythropoiesis being more affected than myelopoiesis (2). This suggests that space anemia may be due to this abnormal erythropoiesis. In our lab, studies on HSCT for space anemia are coupled with studies on the transplantability of HSCs. The ability of the recipient to express donor hemoglobin is indicative of successful transplantability and differentiation of HSCs. In addition, successful expression of donor hemoglobin indicates that HSCT is successful in mitigating anemic conditions in mouse model used. Transplantability of cultured HSCs can be analyzed by using β-thalassemic mice (9). The β-thalassemic mice, which colony we have established in this institution, have been quite useful for us to establish the transplantation procedure and also to evaluate the quality of HSCs for transplantation. Since the hemoglobin molecule of the animal clearly differs from those of wild type mouse and heterozygotes, as analyzed by the cellulose-acetate electrophoresis, the transplantation can be assessed by characterizing hemoglobin species in the transplanted recipients. Thus, transplantability of HSCs grown in RWV system can be analyzed by the β-thalassemic mouse transplantation system. Regarding the β-thalassemic mouse, it is noteworthy that despite the difference in basic mechanisms between the β-thalassemia and spaceflight, there are uncanny similarities in their phenotypes. Namely, the β-thalassemic mouse shows abnormal red cell morphology, reduced hemoglobin concentration and hematocrit, decreased body weight and size, and brittle bones. It may be that the common underlying cause is a hypoxic condition in the body due to reduced hemoglobin concentration, which both disorders display. Thus, this mouse offers a good test model for HSCT, and the protocol derived thereof could well be relevant to space-caused disorders.

HSCT for Muscle Loss

Emerging reports indicate an extraordinary plasticity of HSCs; namely the HSCs, the so-called adult stem cells, can differentiate not only to all types of blood cells but also to muscle, skin, liver, neuronal cells, and possibly bone (10-18). According to Blau et al., as much as 15% of muscle cells could be derived from the transplanted HSCs in normal mouse (17). With regard to bone repair, Cobbs' group showed that not only a fractured bone but also completely disconnected bone gap was repaired by bone marrow derived mesenchimal stem cells (12,18). If this holds true in space, the HSCs could be useful to countermeasure various space-caused symptoms, especially muscle and bone losses (19). Since one of
the aims of our HSCT is to maintain the homeostasis of muscles and bones, as in hematopoiesis above, during long-duration space missions such as Mars exploration, our working hypothesis is that combined with exercise, periodic autologous HSC transplantation might prevent muscle and bone losses of the astronauts during the long-term exposure to 0/μ G, the differentiating HSCs contributing to the repair of these atrophying tissues. We are investigating these possibilities, using a mouse hindlimb suspension unloading model (20). Since this model is frequently used to simulate astronauts’ bone and muscle loss in space, as well as bed-rest patients on earth, information obtained from this investigation may shed light for the countermeasures. Our experimental design involves the use of transgenic (tg) mice which harbor ubiquitously expressing β-galactosidase (LacZ) gene (21) or green fluorescent protein (GFP) gene (22). The HSCs are prepared from these mice and being transplanted to isologous wild type mice that are hindlimb suspended. If the LacZ-HSCs differentiated to muscles and bones, then examination of these tissues for β-galactosidase expression, which can be detected by X-gal (23), blue-color staining, would signify the possibility. Similarly, GFP-HSC can be monitored by the fluorescence emission. While these are the initial studies, more refined anatomical/histological examinations would be necessary to ensure the actual integration of grafted cells to existing tissues. In space situation, our hope is that the earth-programmed HSCs would either form new muscle cells of ground type or fuse to the existing cells to make ground type fibers. As to the frequency of HSC transplantation, the interval could be determined by the rate of muscle fiber transition from slow to fast type (19, 24, 25). The incoming HSCs should prevent this remodeling and thus, this myosin heavy chain (MHC) isofrom change can be the determining factor for frequency.

While it is premature to speculate the contribution of HSCs for repair of bone loss and muscle loss in space, the participation of HSCs for needed repair is apparent from the above reports. In addition, the localization of HSCs to bones and muscles might in the future make it possible to perform HSC-mediated ex vivo gene therapy in space (6,7,27), using insulin-like growth factor 1 (IGF-1) gene which would promote growth of bones and muscles (26) in an autocrine/paracrine fashion. A few words need to be added on the muscle derived stem cells (MDSC), which subject is currently actively pursued by several investigators (28-30). Although we are also working on the MDSC in a mouse system, reproducing preplating methods of Huard’s laboratory (29), there may be a potential difficulty of this approach to space program because of the invasive operations needed to prepare MDSC: namely, muscle specimens from the astronauts have to be obtained before the flight. Unrelated individual’s MDSC would result in graft-vs-host-disease. Thus, the MDSC approach may not be applicable to the space-based stem cell therapy, at least at the current level of technology. Compared with this situation, hematopoietic stem cells can be prepared from the astronauts’ blood samples, as is commonly done.

HSCT for Immunodeficiency

Studies on hematopoiesis in space using human HSCs (CD 34+ cells) indicate a decrease in both erythropoiesis and myelopoiesis. This decrease in myelopoiesis can then lead to decrease immunity at microgravity conditions. Other studies have indicated alterations of several immunological parameters, including leukocyte blastogenesis, cytokine production and leukocyte subset distribution. Since HSCs have the potential to differentiate into all types of blood cells, the hematopoietic stem cell therapy (HSCT) should be able to mitigate this disorder. Since one of the aims of our HSCT is to maintain the homeostasis of immunity during long-duration space missions such as Mars exploration, our working hypothesis is that periodic autologous HSC transplantation might prevent immunodeficiency of the astronauts during the long-term exposure to 0/μ gravity: the differentiating HSCs contributing to the alteration of immune parameters. We are investigating these possibilities using a mouse hindlimb suspension unloading model. Since this model is frequently used to simulate effects of space flight on physiological changes of the body, information obtained from this investigation may shed light for the countermeasures. Our experimental design involves the use of wild type (C57BL) mice which are intraperitoneally infected with E.coli harboring the plasmid, pCMV.SPORT-β-gal. Examination of the tissues for β-galactosidase expression, which can be detected by X-gal, blue-color staining, would signify the level of immunity of host system: blue-color staining is inversely proportional to the ability of mice to eliminate the bacteria; the more blue-color staining, the less immunity. The second stage would then be to mitigate this immunodeficiency via HSCT. These studies would lead to cancer immunotherapy in the
future. It is reported that cosmic radiation causes chromosomal mutation which may lead to cancer. The HSCT could help boost immune system and supply those immunological parameters necessary to eradicate cancerous cells.

MATERIALS AND APPROACH

Experimental animals:
β-thalassemic mouse, C57BL/6Hbb<sup>b</sup>/Hbb<sup>b</sup>: the breeding pairs were purchased from the Jackson Laboratory, ME and thereafter bred in this institution to establish a colony. Breeder pairs for LacZ-mouse, B6;129S-Gtrosa26 and GFP-mouse, C57BL/6-TgN (ACTbEGFP)10bs were also purchased from the Jackson Laboratory and bred in this institution. The latter two mice express the respective reporter genes ubiquitously, except for erythrocytes and hair in the GFP-mouse. The animals were handled and experimented according to the protocols of the Howard University IACUC and IBC.

Bacteria:
A weak strain of E. coli (DH5a) was purchased from Sigma-Aldrich. The bacteria was transformed and maintained on agar/ampicillin plates until use.

Hindlimb suspension mouse model:
Mice were suspended with a head-down tilt of approximately 15°, with no load bearing on hind limbs. This induced fluid shifts to the head, similar to that experienced by astronauts in space flight.

Experimental infection:
For infection studies, samples of transformed E. coli were inoculated into 10mL of LB Broth containing 50µL of ampicillin. This medium was grown overnight at 37°C to late log phase. Growth curve patterns of the bacteria were prepared by plotting absorbance readings at wavelength 800nm versus corresponding bacterial counts (samples diluted 1x 10<sup>6</sup>) on agar plates; these were expressed as colony forming units.
A sample of bacterial suspension was diluted (1x 10<sup>6</sup>) and plated on bacto-agar/ ampicillin plates to determine actual dose of bacteria infected into each mouse. Each mouse received 250 µL of overnight bacterial culture administered through the intraperitoneal route.

HSC transplantation (HSCTP):
The transplantation was carried out according to the procedure described (7,35). For LacZ-mouse, strain 129S was used as the recipient to prevent GVHD. While our routine method of HSC injection is through ocular vein, intramuscular injection to thigh is also being tried, aiming to deliver HSCs directly to leg muscles and bones.

Hemoglobin typing by cystamine-cellulose acetate electrophoresis:
Approximately 0.1 mL of peripheral blood was collected from the retroorbital sinus of mice using a 2 mg/mL Na-heparin as an anticoagulant. Blood cells were lysed with 2 vol. of 'Hb Elution Solution' (ISOLAB; 0.05% KCN, 1% Triton X-100, Na-azide) and the sample reacted with an equal volume of cystamine solution [66.7 mM cystamine, 1.33 mM dithioerythritol, 0.1 M (NH₄)OH]. The samples were run on a cellulose acetate electrophoresis (7,36).

Lac Z staining Procedure:
The HSCs in culture were harvested by centrifugation, fixed with formalin/glutaraldehyde and stained with X-gal-Fe cyanide solution (23). The differentiation of HSCs to muscles was assessed by the Lac Z marker. Harvested tissues were stained according to the procedure of Schmidt et al. (21).

Bacterial organ load studies:
Spleen, liver, kidney and intestines were aseptically removed from dead mice and washed with sterile PBS. The tissues were then stained and observed.

RESULTS

HSC transplantation to β-thalassemic mice

1) β-thal hemoglobin (Hb) types: When hemoglobin is treated with cystamine, the chemical undergoes disulfide interchange reaction with hemoglobin, adding extra positive charges (as cysteamine) to Hbs (36). Since the mouse β<sub>minor</sub>-globin has an additional cysteine residue in relation to β<sub>single</sub>, the Hb<sup>d-minor</sup> is clearly separated from Hb<sup>single</sup> by cellulose acetate electrophoresis after the reaction with cystamine. The Fig. 5A shows the pattern of Hb species of our β-thal mice by this method: the wild type C57BL/6J gives one band (hemoglobin single, Hb<sup>+</sup>), while the heterozygote (C57BL/6Hbb<sup>b</sup>/Hbb<sup>b</sup>) has two bands, Hb<sup>single</sup> and Hb<sup>d-minor</sup> The β-thalassemic mice showed only one band: Hb<sup>d-minor</sup>.
hemoglobin species in the recipients. The longer period of post-transplantation results stronger donor hemoglobin bands (compare 3 weeks vs. 5 weeks).

**Genetic marking of HSCs**

In order to trace the transplanted HSCs in the hindlimb suspended mice, HSCs were designed to be genetically marked, using reporter genes. We have attempted two methods: one is to transfect HSCs with a plasmid harboring β-galactosidase (β-gal) reporter gene, and the other is to isolate HSCs from β-gal transgenic mice. Fig. 6A shows the β-gal plasmid-transfected HSCs, which were subsequently stained by X-gal staining procedure, while Fig. 6B shows the HSCs isolated from the transgenic mice, similarly stained with X-gal. The transfection resulted in more than 50% of HSCs to be marked with β-gal, while 100% of HSCs from the transgenic mice were positive in X-gal staining.

**Transplantation of the marked HSCs for HSCT for muscle loss**

To initiate the HSC therapy for bone and muscle losses, we set up the mouse hindlimb suspension unloading system in this laboratory. Currently, β-galactosidase-marked HSCs are being transplanted to the isologous hind limb suspended mouse and differentiation of the HSCs to muscles are
investigated by X-gal staining procedure. GFP-marked HSCs will be also used in the future. Effect of exercise on the HSC engraftment and differentiation is being investigated. If the engraftment/differentiation were proven, further studies, such as integration/participation of HSCs to existing muscle structure, will be conducted. Effect of HSCT and exercise on the prevention of slow-to-fast-type muscle fiber is under investigation utilizing myosin heavy chain (MHC) isoform analysis (24,25,37).

Fig. 7. Transplantation of β-gal-HSCs to a hindlimb suspended mouse. HSCs from a β-gal transgenic mouse were isolated and expanded, and thereafter about 2x10^3 cells in 0.25 ml of HBSS was injected to thigh and gastrocnemius regions of the right leg of a hindlimb suspended mouse. Two days later, the mouse was sacrificed and various tissues harvested and stained by X-gal stain. Note that segments of the large and small intestine (a) were strongly stained, while the portions of left leg (b) and right leg (c) were positive by the stain. Pictures were taken by Nikon Coolpix 5000.

Fig. 8. Tissues from Fig. 7 C were sectioned and stained by hematoxylin-eosin. Note that the HSCs were infiltrating into muscle fibers.

Genetic marking of E.coli

To initiate the HSC therapy for bone and muscle losses, we set up the mouse hindlimb suspension unloading system in this laboratory. As a test for level of immunity via organ load studies, it was necessary to transform the E. coli bacteria with pCMV.SPORT-β-gal, a plasmid that was easily detectable when stained with X-gal, as shown in Fig. 9.

Fig. 9. Transformation of E. coli with pCMV.SPORT-β-gal. Note the blue color of the E. coli indicates that the bacteria was successfully transformed. Bacteria was plated on X-gal labeled ampicillin-agar plates. Plates were incubated overnight at 37°C.

Transplantation of the HSCs for HSCT for immunodeficiency

Examination of hindlimb suspended, E.coli [β]-gal injected tissues, indicates an increase in the intensity of blue-color when tissues are stained with X-gal as compared to control mice. This indicates a decrease in the ability of hindlimb suspended mice to eliminate E.coli and therefore a decrease in immunity of hindlimb suspended mice. Examination of hindlimb suspended mice injected with E.coli-β-gal and subsequently treated with donor HSCs indicate an increase in agility and alertness of mouse plus a decrease in blue-color when tissues are stained with X-gal as compared to hindlimb suspended mice that are not treated with HSCs.
The HSCT for immunodeficiency necessitates the ability to quantify the level of immunity in the mice model. In this lab, we have decided to quantify the level of immunity by calculating the number of E. coli colony forming units in the blood; with level of immunity being inversely proportional to the number of colony forming units in the blood.

Fig. 10. Effect of hindlimb suspension unloading and HSCT on the resistance to LacZ-E. coli infection. 0.25mL of β-gal-E. coli was injected intraperitoneally to A: control mouse; B: 2 weeks hindlimb unloaded mouse; and C: 2 weeks hindlimb unloaded and HSC-treated mouse (2x 10^3 HSCs in 0.25mL of HBSS, i.p. one day before the infection). Eight hours after the infection, tissues were ar rested from all the mice and stained by X-gal staining method.

Quantifying E. coli in blood stream

As shown in Fig. 11, each colony forming unit is represented by a blue circle on agar plates labeled with X-gal. Preliminary results are concurrent with the results from whole mount tissue staining, show the greatest number of colonies for hindlimb suspended mice than hindlimb suspended-HSC treated mice.

DISCUSSION

We have hypothesized that the hematopoietic stem cell therapy (HSCT) might be effective in maintaining health condition of astronauts during long-duration space missions, such as Mars exploration. While there are several known symptoms which astronauts experience in 0/µ G, we focused on space anemia, muscle loss and immunodeficiency. To formulate the relevant techniques and protocols, two animal models are being used in the ground-based experiments: β-thalassemic mouse and mouse hindlimb suspension system. The goal is to test the ability of the HSCT to mitigate the hematological disorders of the β-thalassemic mouse as well as the ability of the HSCT to treat muscle loss and immunodeficiency. Another major goal of the β-thalassemic mouse model is to test the transplant ability if HSCs as well as the quality of HSCs grown in the NASA Rotating Wall Vessel (RWV) culture. In this paper, some of the success of the transplant ability of HSCs thereby correcting β-thalassemic mouse as studied by the hemoglobin change is presented (Fig. 7B). Successful cure of β-thalassemia by HSCT may normalize various hematological parameters. These would include white blood cell counts, lymphocyte counts, hematocrit, reticulocytes counts, RBC volume (MCV), and hemoglobin content (MCHC) (38). Thus, the HSCT protocols derived from this animal model would be highly relevant to the HSCT for space-caused hematological abnormalities.

With regard to the study on efficacy of HSCT for muscle loss, the current goal is to delineate participation of HSCs in repair/prevention of muscle and bone losses in the hindlimb suspended mouse. The reporter gene marked HSCs are useful to trace transdifferentiation in the transplant recipient. We have successfully marked the HSCs with β-galactosidase expression by two methods, transfection and transgenic mouse, which are presented in Fig. 6A,B. The transfection method will
be useful for marking human HSCs in the future clinical trials. Further physicochemical analyses for the actual countermeasure will be performed. These will include: 1) Measurement of muscle weight; 2) Study of prevention of MHC transition by HSCT; and 3) Measurement of muscle strength. In addition, using the hindlimb suspension model, effect of exercise on the HSCT for muscle loss can be investigated. Combined with proper exercise regimen, periodic HSC transplantation might prevent muscle losses.

It is argued that since stress has been shown to alter immunologic parameters, stress significantly contributes to the decrease in immunity observed in hindlimb suspended mice. However results from this experiment indicating that HSCT can mitigate the immunodeficiency, provides evidence that in fact, stress is not a significant contributor in the decrease in resistance to infection in these mice. These findings are compounded by previous studies comparing the resistance to infection of hindlimb suspended mice and restrained mice, wherein the restrained mice showed no signs of an immunocompromised system. This observation is significant in the validation of the use of the hindlimb suspended system as a ground based model for astronauts in space flight. It provides evidence against those who might argue that the hindlimb suspension system produces stress in mice which is not experienced by astronauts who participate in several activities which acclimatized them to space flight thus reducing the stress they might experience.

Our results have successfully indicated that not only do hindlimb suspended mice suffer an immunocompromised system, but that HSCT has the capability of mitigating this immunodeficiency. It also provides evidence that the immunodeficiency was due to either inhibition of stem cell growth or decrease hematopoiesis at micro gravity conditions. More significantly since the HSCT mitigated the disorder, this proves that the HSCs successfully differentiated at micro gravity conditions, leading to assumption that the decrease in immunity was in fact due to inhibition of stem cell production at micro gravity conditions: an aspect of this research which needs to be investigated further. Finally, since astronauts suffer a decreased immunity similar to that of hindlimb suspended mice, the HSCT protocols derived from this animal model would be highly relevant to the HSCT for immunodeficiency in astronauts. Our current goals are now to quantify the degree of immunodeficiency exhibited in the hindlimb suspended mouse and then to possibly delineate the process of HSCT for immunodeficiency.

FUTURE PLAN

The HSCT necessitates optimizing the frequency with which HSCs should be administered. One immediate goal in this laboratory is to delineate this interval using SDS-polyacrylamide gel electrophoresis (PAGE) which measures changes in muscle fibers (37). In this experiment we will examine two types of muscle fibers that are used in daily activity: slow type and fast type. The slow type which is used for gravity support is changed to the fast type during microgravity exposure (25, 26). By determining the frequency of HSC administration, we need to prevent this conversion. In the future, the HSCT will be applied to long-term bed rest patients to prevent their muscle losses. This, in turn, would lead to prevention of muscle loss in astronauts in space. As for the HSCT for immunodeficiency, exploiting the capability of HSCs to augment host immunity, we will investigate the feasibility of HSCT for cancer. Radiation-caused cancer is a major concern for long-term space exploration. Furthermore, our long-term plan is to develop adeno-associated virus (AAV)-mediated gene therapy in space to countermeasure various symptoms. The AAV-gene therapy for hemoglobin disorders (β-thalassemia and sickle cell disease) is currently being developed in this laboratory, and thus, one of our expertise.

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REFERENCES


