MASTER

TECHNICAL PROGRESS REPORT

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V. Incubated Spleen and Liver

Cells to Minimize Secondary Disease

in Allogeneic Radiation Chimeras

Mortimer M. Bortin, M.D.
Principal Investigator

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The May and Sigmund Winter Research Laboratory

Mount Sinai Hospital

948 North Twelfth Street

Milwaukee, Wisconsin 53233

and

The Departments of Medicine, Biostatistics and

Surgery, Marquette Medical School, Inc.

PROJECT ABSTRACT

This research was performed in an effort to improve stem cell viability and the radioprotective ability of incubated liver and spleen cell mixtures and to test whether incubated liver and spleen cell mixtures minimized secondary disease in mice.

SCIENTIFIC BACKGROUND

The clinical potential of human bone marrow transplantation has not been realized mainly because of failure of successful engraftment and the post-transplant complication of secondary disease (1). Failure of engraftment appears to be related to inadequate immunosuppressive preparation of the recipients with resultant host versus graft rejection of the transplanted bone marrow. Secondary disease encompasses immune inadequacy of the recipients with resultant susceptibility to infection (2) and graft versus host (GVH) disease, the immunologic attack against the tissues of the host by the immunologically competent transplanted cells (3). In general, these two problems of immune inadequacy and GVH disease appear to be inversely related. Attempts to improve the immune status worsen the GVH reaction; efforts to minimize GVH disease tend to maximize immune inadequacy. Recent efforts in our laboratory to overcome these problems have met with some success both in rodents (4, 5, 6), dogs (7), and man (8).

In 1968, we reported (9) that mouse perinatal liver hematopoietic cells were immunologically incompetent against transplantation antigens. This study used the Simonsen GVH spleen weight-gain assay and showed that liver cell suspensions obtained from perinatal C57BL/6 mice did not cause splenic

enlargement when given to B6D2F₁ mice. The morphologic appearance of the nucleated cells in these liver cell suspensions was predominantly erythroid. Although the absence of a GVH reaction was probably due to a lack of lymphoid cells in the inoculum, we recognize that other explanations were possible. Our method of processing perinatal liver (10) intentionally disrupts the hepatic parenchymal cells. Mandel, Monaco and Russell (11) have reported that a factor in liver homogenate interfered with the reactivity of cell-free splenic transplantation antigens. It seemed possible that the breakdown of liver cells during processing may have released ^a "liver factor." This "liver factor" could have interfered with the reactivity of cells in the parental liver cell suspensions and prevented splenomegaly in the hybrid hosts. Cosgrove, Upton, Popp and Congdon reported reduced mortality in sublethally x-irradiated F₁ hybrids when parental spleen cells were first incubated with F₁ host liver homogenate (12). In view of our findings and these reports, we tested C57BL/6 liver cell suspensions for a factor which might reduce the reactivity of adult parental spleen cells, which we knew were capable of provoking a positive GVH reaction.

Evaluation of GVH disease in B6D2F₁ mice was done by growth curves, weight, survival and Simonsen spleen weight-gain assays (4). Mixing and incubating adult spleen with liver cell suspensions from 5-day-old C57BL/6 donors was successful in preventing GVH reactions by all of these tests in this "hot" GVH parent-F₁ hybrid combination. Incubating immunologically competent C57BL/6 spleen cells alone resulted in a significantly reduced, but still strongly positive, GVH reaction.

This Technical Progress Report describes the results to date of our efforts

to improve stem cell viability and the radioprotective ability of incubated liver and spleen cell mixtures and to test the ability of incubated liver and spleen mixtures to minimize or to prevent secondary disease in mice.

SCIENTIFIC SCOPE

Inbred strain-A/J and CBA/J mice were used in this study. Pregnancies were timed at, and all mice were obtained from, The Jackson Laboratory, Bar Harbor, Maine. The radiation conditions and methods for this project have been detailed in previous communications from our Laboratory (4,5,10,15,16).

I. Endogenous Spleen Colony Formation

Groups of 30 adult female CBA mice were irradiated with 450, 500, 550, 600, 650, 700 and 750 r total body radiation (TBR). No cells were administered. Eight or 9 days following irradiation, the mice were sacrificed, their spleens removed and the spleen colonies counted. A dose of 750 r was found to be sufficient to virtually eliminate endogenous spleen colony formation (Figure 1). A dose of 850 or 950 r was used for all subsequent radiations.

II. Colony-forming Ability of Incubated Liver and Spleen Cell Mixtures

A. Liver Hematopoietic Cells at 4°C

Thirty CBA mice received 850 r TBR and 10^6 liver cells from 5-day-old CBA mice.

B. Spleen Hematopoietic Cells at 4°C

Thirty CBA mice received 850 r TBR and 10^6 spleen cells from adult CBA mice.

C. Liver and Spleen Cell Mixtures at 4°C

Thirty CBA mice received 850 r TBR and 5×10^5 liver cells from 5-day-

Figure 1. Endogenous spleen colony formation in CBA/J mice following whole body x-irradiation. Each point represents 24-30 spleens. Spleen colonies were counted as unknowns by 2 observers.

old CBA mice and 5×10^5 spleen cells from adult CBA mice which were mixed and incubated together for one hour at 4°C .

D. Liver and Spleen Cell Mixtures at 37°C

Thirty CBA mice received 850 r TBR and 5×10^5 liver cells from 5-day-old CBA mice and 5×10^5 spleen cells from adult CBA mice which were mixed and incubated together for one hour at 37°C .

The host mice were sacrificed and their spleens removed 8 or 9 days following irradiation and cell inoculation. The spleens were placed in Bouin's solution. Spleen colonies were counted as unknowns by two observers under the ten power objective of a dissecting microscope. The results are shown in Table 1.

Group	Recipients	Tissue	Cells	Temp.	CFU Ratio ^a
A	30	Liver	10^6	4°C	1.412
B	30	Spleen	10^6	4°C	1.330
C	30	Liver & Spleen	5×10^5 & 5×10^5	4°C	1.288
D	30	Liver & Spleen	5×10^5 & 5×10^5	37°C	1.305

^a Number of spleen colonies per 10^5 cells administered.

TABLE 1. Spleen colony formation in groups of 30 CBA mice after 850 r TBR and inoculation of liver, spleen or combined liver and spleen cell suspensions that had been incubated for 1 hour.

There was no significant difference between the CFU ratios of liver and spleen cell mixtures incubated at 4°C and 37°C .

III. Radioprotective Ability of Syngeneic Incubated Liver and Spleen Cell Mixtures

A. Spleen and Liver Cells at 4°C

Five groups of 15 adult female CBA mice were given 950 r TBR and then received graded doses of a mixture of equal numbers of syngeneic adult spleen and 5th day postnatal liver cells which were maintained for 1 hour at 4°C. The cell doses for each tissue were 1, 2, 4, 8 and 16×10^5 . The mice were observed for thirty days and mortality curves were constructed. An estimate was made of the number of cells required to permit 50% of the mice to survive 30 days. The experiment was then repeated with four groups of 15 adult female CBA hosts. The cell doses of each tissue used were 6, 8, 10 and 12×10^5 . The data from both sets of experiments were combined and mortality curves constructed. A cell dose of $8.5-9 \times 10^5$ was found to result in an LD_{50/30}.

B. Spleen and Liver Cell Mixtures at 37°C

The above sequenced experiment was repeated with the incubation conditions changed to 37°C. The cell dose for both parts of the experiment was the same as in III. A. A cell dose of $9.5-10 \times 10^5$ of each tissue incubated at 37°C for one hour was estimated to provide an LD_{50/30}.

IV. Improved Viability of Incubated Liver and Spleen Cell Mixtures

A. Nigrosin Dye Exclusion

Equal numbers of liver cells from 5-day-old CBA mice and spleen cells from adult female mice were mixed and incubated at 37°C for 1 hour.

The effects on viability of various solutions with and without fetal calf serum in the presence of various concentrations of CO₂ were tested using a factorial design (Fig. 2). Twenty-seven incubation conditions were tested. Cell viability was screened using Nigrosin dye exclusion and nucleated cell counts of the mixture before and after incubation. The results obtained from the 27 incubation conditions did not lend themselves to formal statistical analysis for the determination of an optimal set of incubation conditions. However, the data suggested that cells incubated using TC 199 in an atmosphere containing 7% CO₂ with 12 1/2% or 25% fetal calf serum had best survival. This finding is in accord with the report of Smith and McKinley (14).

In Experiments IV. B and C, cell suspensions were prepared and incubated using TC 199, 7% CO₂ and 12 1/2 or 25% fetal calf serum.

B. Colony-forming Ability

Two groups of 30 CBA mice received 850 r TBR and 5×10^5 liver cells from 5-day-old CBA donors and 5×10^5 spleen cells from adult CBA donors which were incubated together at 37°C, under the conditions described above. The results of tests of exogenous spleen colony formation in these mice are not yet available. The results will be compared with those found in Experiments II. C and D.

C. Radioprotective Ability

Two groups of 30 CBA mice were given 950 r TBR and then received 10×10^5 liver cells from 5-day-old CBA donors and 10×10^5 spleen cells from adult CBA donors. The cell mixtures were incubated together at

Figure 2. Factorial design used to screen various conditions affecting viability of incubated cell mixtures. * = incubation conditions selected.

37°C under the conditions described in IV. A. These mice are presently being observed and thirty days after initiation of these experiments mortality curves will be constructed and compared with those found in Experiment III. B.

V. Incubated Liver and Spleen Cell Mixtures to Minimize Secondary Disease

All of the preceding experiments and experiments A and B in this section were designed to test levels of viability of liver and spleen cell mixtures. These tests were all of a functional nature. An effort was made to "focus in" on the incubation conditions which would best preserve hematopoietic stem cell function. (However, the prime objective of this research was to determine whether incubated liver and spleen cell mixtures could be used for hematopoietic reconstitution across strong immunogenetic barriers without producing death from secondary disease. Sections V. C and D, describe the key experiments which will test this hypothesis.) The results of these experiments are not available for this Progress Report. All of these final experiments will be started in March, 1970.

A. Syngeneic Liver and Spleen Cell Mixtures at 4°C

Sixty adult female CBA mice will receive 950 r TBR plus a mixture of 10^7 liver cells from 5-day-old CBA donors and 10^7 spleen cells from adult female CBA mice. These cells will be mixed and incubated together for 1 hour at 4°C.

B. Syngeneic Liver and Spleen Cell Mixtures at 37°C

Sixty adult female CBA mice will receive 950 r TBR plus a mixture of 10^7 liver cells from 5-day-old CBA donors and 10^7 spleen cells from

adult female CBA mice. These cells will be mixed and incubated together for 1 hour at 37°C . Incubation conditions will be those found to provide highest CFU ratios and greatest radioprotection in Experiments IV. B and C.

C. Allogeneic Liver and Spleen Cell Mixtures at 4°C

Sixty adult female CBA mice will receive 950 r TBR plus a mixture of 10^7 liver cells from 5-day-old strain-A mice and 10^7 spleen cells from adult strain-A donors. The cell mixtures will be incubated together for 1 hour at 4°C .

D. Allogeneic Liver and Spleen Cell Mixtures at 37°C

Sixty adult female CBA mice will receive 950 r TBR plus a mixture of 10^7 liver cells from 5-day-old strain-A mice and 10^7 spleen cells from adult strain-A donors, which have been incubated together for 1 hour at 37°C . Incubation conditions will be those found to provide highest CFU ratios and greatest radioprotection in Experiments IV. B and C.

All mice treated in section V. will be followed for 100-day survival.

SIGNIFICANCE

Previous studies in our Laboratory (5,6) have established levels of survival to be anticipated when lethally irradiated CBA mice receive unincubated post-natal liver cells or spleen cells from syngeneic or strain-A donors. Approximately 90% of lethally irradiated CBA mice survived 100 days when given 2×10^7 liver cells from 5-day-old CBA donors or 2×10^7 spleen cells from adult CBA donor (syngeneic transplants). Approximately 50% of lethally irradiated CBA mice survived 100 days when given liver cell suspensions from 5-day-old

strain-A donors (allogeneic transplants). All lethally irradiated CBA mice died within 33 days of, what appeared to be, acute GVH disease when injected with 2×10^7 spleen cells from adult strain-A donors (allogeneic transplants). We anticipate that the CBA mice in group V. C, that will receive allogeneic liver and spleen cell mixtures maintained at 4°C , will die of GVH disease. It is hoped that the mice in group V. D, that will receive allogeneic liver and spleen cells mixtures incubated at 37°C , will have high long-term survival rates. If, in fact, secondary disease is minimized by incubating immunocompetent lymphoid cells with liver cell suspensions, we would wish to do additional experiments to investigate the mechanism of this effect and to test the level of immune competence of the resultant radiation chimeras.

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