A critical analysis of bone marrow-spleen cell interaction in the immune response
by Donna Gail Sieckmann

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements of the degree of MASTER OF SCIENCE in Microbiology
Montana State University
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Abstract:
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Spleen dilutions from normal SPF mice could not interact synergistically with bone marrow, but could interact with normal thymus cells. Spleen dilutions from immune mice or older conventionalized mice were able to interact synergistically with bone marrow. The appearance of the synergistic cell in the immunized spleen followed that of the memory cell population. It was concluded that the size of the antigen reactive cell population of the spleen could be.- altered by immunization, exposure to cross-reacting antigens and/or aging of the animal.

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Date August 5, 1970

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DONNA GAIL SIECKMANN

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ABSTRACT

The spleen-bone marrow cell interaction phenomenon was studied in three strains of mice, using various experimental parameters. Lethally irradiated mice were reconstituted with either spleen, thymus, bone marrow, or combinations of these cell suspensions and immunized with sheep erythrocytes or bovine gamma globulin. The immune response was measured by Jerne plaque spleen assay or immune elimination.

The immune response in spleen or bone marrow reconstituted animals was dependent upon two cell interactions taking place. However, low doses of spleen and bone marrow alone were not able to immediately reconstitute an animal's immune potential.

Spleen dilutions from normal SPF mice could not interact synergistically with bone marrow, but could interact with normal thymus cells. Spleen dilutions from immune mice or older conventionalized mice were able to interact synergistically with bone marrow. The appearance of the synergistic cell in the immunized spleen followed that of the memory cell population. It was concluded that the size of the antigen reactive cell population of the spleen could be altered by immunization, exposure to cross-reacting antigens and/or aging of the animal.

INTRODUCTION

The role of lymphoid cells in the development and maintenance of an animal's immunological competence has been well documented during the last two decades. However, data which reveal the complexity of the mechanism of antibody formation at the cellular level have only recently been provided. There has been evidence suggesting that cell-cell interactions are involved in the development of an immune response (16, 21, 11, 12). Fishman (11) was first to show that macrophages, which had ingested bacteriophages, produced an RNA species that could induce neutralizing antibody formation in nonimmune lymphocytes. Ford, Gowans, and McCullagh (12) showed that peritonial macrophages, which had ingested sheep erythrocytes (SRBC), could induce specific antibody formation by culturing them with thoracic duct lymphocytes.

In 1966, Clamen, Chaperon, and Triplett (4) presented more direct evidence for the involvement of two cell types in antibody production. They showed that when the immune potential of lethally irradiated mice was reconstituted with both thymus and bone marrow cells, the cellular response to SRBC, as measured by foci of antibody production in the spleen, was several fold greater than the response which would have been expected had the reconstituting effect of thymus and marrow cells been only additive. The results clearly

showed that a synergistic interaction between the two cell populations had taken place. This phenomenon of thymus-bone marrow synergism has since been confirmed repeatedly with SRBC as antigen (10,21) and also with protein antigens (3,31).

With the development of the Mishell-Dutton technique for immunization of dissociated spleen cells *in vitro* (24), it was soon discovered that three cell types were involved in the *in vitro* response to SRBC: a glass-adherent cell, now thought to be the macrophage, and two nonadherent cells (25). The adherent cell was found to be necessary for an *in vitro* primary response to SRBC. However, this cell is present and functional in the lethally-irradiated animals used in *in vivo* studies of cell interaction. The exact role of the adherent cell is presently being debated.

The functions of the nonadherent cells have been more successfully investigated. By use of F_1 hybrids and anti-H2 isoantisera, it has been demonstrated that the bone marrow cell or cells derived from the marrow produce antibody (8, 22, 30). These are the plaque forming cells (PFC) of the Jerne hemolysis-in-gel assay for antibody-producing cells and are also the cells responsible for the different classes of antibodies produced during the immune response (7).

The other nonadherent cell, the thymocyte or a cell derived from the thymus, has now been termed the antigen reactive cell (ARC).

It has been shown (21, 29) that this cell proliferates after contact with the antigen and then interacts with antigen and a percursor of the PFC (P-PFC) to cause differentiation of the P-PFC into the PFC. By statistical analysis, it has been shown (29) that after the initial contact with antigen, the ARC undergoes 6 to 10 cell divisions, producing 80 to 800 progeny ARC, each of which is able to interact with approximately 150 PFC. These figures, which depict a mushrooming of the PFC population as the result of antigen contact with only one ARC, help to explain the magnitude of the response obtained after the synergistic interaction between these two cell populations.

Cell-cell interactions can be most profitably studied by use of thymus and bone marrow, which contain relatively pure populations of the ARC and P-PFC, respectively. The premium effect, shown by Celada (2), or the dilution effect, as it is called by Talmage and co-workers (30), and studies by Mosier and Coppleson (25) indicate that cell interactions can also take place between two lymphoid cells in the spleen, namely, a thymus-derived ARC and a bone marrow-derived P-PFC. These investigators showed that the response to antigen was a nonlinear function of the spleen cell dose given to lethally-irradiated anumals or in *in vitro* culture with antigen. A 50 fold increase in the munber of spleen cells given was accompanied by a 2500 fold increase in the response of the irradiated animal (26).

Talmage and co-workers furthermore demonstrated that a subfraction of unprimed spleen gave no response alone, but could give a synergistic response to SRBC if given with bone marrow (26).

On the other had, Clamen and co-workers (5) did not detect any enhancement of the immune response of spleen cells by bone marrow. Radovich (27) also reports that spleen-bone marrow synergism does not occur in all strains of mice tested.

In our laboratory, preliminary experiments were run in attempt to show spleen cell-marrow cell synergism in order that it might later be applied to the study of immunologic tolerance. A synergistic effect could not be shown. The experiments which followed, and that are reported below, sought to determine the cause for the discrepancies between reports in the literature on spleen-bone marrow cell interactions.

MATERIALS AND METHODS

Mice. Young adult C57Bl (H-2^b), Balb/C (H-2^d), and LAF_1/J (C57L/J x A/HeJ) inbred mice, 6 to 12 weeks old, were used in the experiments. Specific pathogen free (SPF) C57Bl and Balb/C stock breeders were obtained from Dr. J. J. Trentin, Baylor University Medical School, Houston, Texas, in 1968, and have since been maintained by brother-sister matings under SPF conditions. LAF_1/J mice were obtained at the age of 6 weeks from R. B. Jackson Memorial Laboratories, Bar Harbor, Maine, and were housed in the SPF animal quarters upon arrival. All cages, watering bottles, and San-i-cell bedding were autoclaved before use. Mice received Wayne Sterilizable Lab Chow or Autoclavable Purina Laboratory Chow 5010, which had been sterilized, and acidified-chlorinated water (20) *ad libitum*. All mice remained in the SPF animal quarters until removal for experimental use.

Antigens. Sheep (SRBC) and horse (HRBC) blood in Alsever's solution were obtained from Colorado Serum Co., Denver, Colorado, and washed three times in sterile 0.85% NaCl (saline) before use in immunization, serum titrations, or Jerne plaque assay.

Lyophilized bovine gamma globulin (BGG, Cohn Fraction IV) was obtained from Immunology, Inc, Glyn Ellyn, Illinois. The BGG was dissolved in saline and used for immunization either in an aggregated form, prepared by heating for 20 min at 70° C, or as an emulsion

consisting of equal volumes of Freund's Complete Adjuvant (FCA, Difco, Detroit) and BGG solution. BGG solutions used for isotopic labeling or to induce tolerance (soluble BGG) were freed of aggregated molecules by centrifuging at 40,000 x G for 30 min in a Spinco Model L refrigerated ultracentrifuge with an SW25.1 rotor.

BGG solutions were quantitated by optical density analysis at 280 nm in a Beckman DB Spectrophotometer. The $E_{0.1\%}$ was found to be 1.040 in 0.05M sodium phosphate buffer, pH 7.0, using a solution whose concentration was determined by micro-Kjeldahl nitrogen analysis.

Irradiation. Mice were given 900 rad whole body gamma irradiation using Co-60 Teletherapy units with the following dose rates: 162 R/min at the University of Nebraska Medical Center, Omaha, Nebraska, by Dr. E. O. Jones; 74 R/min at Lincoln General Hospital, Lincoln, Nebraska, by Dr. John McGreer, Jr.; 72 rad/min at St. James Hospital, Butte, Montana, by Drs. L. C. Brewton and L. Hammer. Twelve mice were held fast in a round perforated plexiglass cage (H&H Plastics, Lincoln, Nebraska), designed for irradiation purposes. This cage was positioned in a 19 x 19 cm irradiation field, 60 cm below the source, on top of a turntable (Sieckman Const. Co., Lincoln, Nebraska). In order to achieve uniform irradiation of all animals, the mice were rotated at 2rpm during irradiation. Maximum

backscatter conditions were produced by placing a five-inch thickness of masonite under the cage on the turntable. The mice were held in the cage only for the duration of the irradiation time, which varied between 5 and 15 min, depending upon the source used.

In several experiments a Model M Cs-137 Gammator (M34-1-1074, Radiation Machinery Corporation, Parsippany, Illinois) was used. The mice were irradiated individually at a rate of 195R/min.

The type of source used in any particular experiment will be recorded within the experimental procedure.

Preparation of Reconstituting Cell Suspensions. Donor and recipient mice were generally pre-bled, and when possible, were selected from mice showing no natural SRBC hemagglutinins.

Donor spleens were removed aseptically, placed in Hank's balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, N. Y.), teased apart with forceps, and pipetted gently with a Pasture pipette to produce a single-cell suspension. Large particles were allowed to settle out and the supernate was brought up to 15 ml, quantitated with a hemacytometer, centrifuged at 500 x G in the cold for 10 min, and resuspended in fresh HBSS to the proper cell concentration.

Bone marrow was obtained from femurs and tibias of donor mice. Bones were removed and cleaned of tissue. The marrow was removed by

cutting off the epiphyses and injecting HBSS into the marrow cavity thereby forcing the marrow out of the other end of the bone. A single cell suspension was produced by pipetting gently with a Pasture pipette. Large particles were allowed to settle out and the supernate was quantitated with a hemacytometer, centrifuged in the cold at 500 x G for 5 min, and resuspended in fresh HBSS to the proper cell concentration.

Thymus cell suspensions were prepared by mashing the thymus in HBSS against a fine mesh wire screen with the flat end of a glass syringe plunger. Further processing was the same as that for bone marrow.

Reconstitution of Irradiated Mice. Mice were generally reconstituted at 5 hrs after irradiation. Cell suspensions were mixed and injected intravenously (IV) in a lateral tail vein. In cases of immunization to SRBC or HRBC, the antigen was mixed with and injected with the cell suspension. When BGG was used for immunization, the aggregated form was given intraperitoneally (IP) after reconstitution of the mouse. Experimental mice were maintained on sterile feed and acidified-chlorinated water *ad libitum*. Mice to be assayed by immune elimination were started on 0.1% KI in tap water at the time of reconstitution.

Jerne Plaque Assay. Spleens were assayed for antibody

producing cells by the method of Jerne and Nordin (17). Spleens were removed aseptically, and placed in cold Eagle's minimal essential medium, Hank's base (MEM, Grand Island Biological Co.). After the spleen was teased apart with forceps, cell aggregates were further broken down by gently pipetting with a Pasture pipette. Coarse material was allowed to settle out in a centrifuge tube, and the supernate was removed and plated immediatly thereafter. Either 0.1 or 0.2 ml of the resulting cell suspension or a 1:10 dilution of the suspension was added to an overlay agar mixture at 47°C. The overlay was composed of 1 ml of 1% agarose (L'Industrie Biologique Française S. A.), 1 ml of 2x MEM, and 0.9% NaCl. The overlay was spread in a plastic petri dish, which contained a base agar layer composed of 10 ml of 1.4% Ionagar, No. 2 (Oxoid, Colab Laboratories) in HBSS, to which 500 µg of DEAE-dextran was added per ml of media after autoclaving. Both the overlay and the base agar solutions were filtered after sterilization and adjustment to pH 7 with 7.5% $NaHCO_3$. Double distilled water was used for all solutions.

The plates were incubated at 37°C in a humidified 5% CO_2-95% air incubator for 3 hrs. Development of plaques was accomplished by addition of 1.5 ml of 1:10 guinea pig complement (Difco, Detroit, or Colorado Serum Co., Denver) and incubation at 37°C for 1 hr. Plaques were counted under a dissecting microscope at 100x magni-

fication. Spleen cell suspensions were quantitated with a hemacytometer.

Assay of Immunity to BGG by Immune Elimination: Preparation of Labled Protein. The Chloramine T method of McConahey and Dixon (19) was used to lable BGG with ^{131}I. Iodine-131 was obtained as $Na^{131}I$, biochemical grade, in carrier-free form from Mallinckrodt Nuclear. Chloramine T, 200 μg in 0.05 ml sodium phosphate buffer, pH 7.0 (Eastman Organic Chemicals) was injected into a small stoppered vaccine bottle containing 200 μg soluble BGG in 0.4 ml buffer and 200 μC $Na^{131}I$ in 0.1 ml buffer. The vial was gently agitated on crushed ice for 10 min. Then 0.05 ml (400 μg) sodium meta-bisulfite (Fisher Scientific Co.) was added with a syringe to stop the reaction and to reduce any remaining Chloramine T and free iodine. The BGG was separated from the salts on a 0.9 x 15 cm Sephadex G-50 (superfine) column (15). The column had been prewashed with nonlabled BGG and phosphate buffer. Ten-drop fractions were collected and assayed for radioactivity with a Nuclear Chicago Scintillation Counter-Analyzer Computer (Model 132A) in a well-type sodium iodide crystal Scintillation Detector (Model DS5). Active fractions at the void volume were pooled, and the protein content was assayed by optical density at 280 nm. The pooled fractions were diluted to a concentration of 100 μg protein N/ml. The labled protein was generally used immediately.

Immune Elimination. Each mouse, which had previously been maintained on 0.1% KI in tap water, was given 10 µg ^{131}I-BGG (approx. 0.7 µC) IV. Whole body counts were taken immediately and at various times thereafter. The mouse was held in a small cup, which was placed directly on top of the NaI crystal detector. At least 6400 counts were taken to give values with no more than 3% error. Backround activity was measured with the empty mouse holder in place. Correction for daily variation in machine efficiency and for decay of isotope still present in the animal was made by counting a standard, consisting of a stoppered test tube containing a solution of Na^{131}I. Correction for counting efficiency was necessary, due to fluctuations in line voltage. Results are expressed as the percentage of initial activity remaining in the animal at a certain time after injection of the labled material. For sample calculations, see the appendix.

Serum Titrations. Sera were titrated using a microtitrator system (CookeEngineering Co., Alexandria, Va.). Twenty-five µl of serum was diluted in two-fold dilutions, 1:2 to 1:4096, in modified barbital buffer (1). Then 25 µl of 0.5% SRBC in 1% normal, absorbed, heat inactivated mouse serum in buffer was added. Hemagglutinin assays were incubated at room temperature for 1 hr and then overnight at 4°C. Hemolysin assays were held 10 min at room temperature

before adding 25 µl of a 1:20 dilution of guinea pig complement in buffer. Incubation for 1 hr at 37°C and overnight at 4°C completed the hemolysin assay.

RESULTS

Irradiation and Hematopoietic Reconstitution. A preliminary experiment was conducted to test the effectiveness of irradiation and reconstitution of the strain of mice to be used in these studies. C57Bl female mice were given either 600 or 900 rad whole body irradiation at the University of Nebraska Medical Center. Syngeneic bone marrow was administered to several of the irradiated mice either IV or IP. All mice receiving 600 rads survived and showed very little weight loss (Table I). A dose of 900 rad proved lethal for nontreated mice. The time of death indicated that it was due to hematopoietic failure and not damage of the intestial tract. Death could be prevented by reconstitution with 2×10^7 syngeneic bone marrow cells. Either route of injection proved satisfactory for this number of bone marrow cells. A dose of 900 rad was used in subsequent experiments.

Assay of Immunity to BGG by Immune Elimination. In anticipation of studying cell interactions involving a soluble protein antigen, preliminary experiments were run to determine the practicality of using immune elimination of ^{131}I-BGG as an assay for immunity to BGG.

A. Immune and Nonimmune Elimination. The average elimination pattern of 8 normal C57Bl mice is shown in Fig. 1 by curve A. The 10 µg ^{131}I-BGG given IV was eliminated with a half-life of 3.2 days in the linear portion of the curve.

Curve B represents the average of three mice which had been

TABLE I. The Effect of Irradiation and Hematopoietic Reconstitution

Mouse Number	Dose (rad)	Treatment[a]	Body Weight (gms) Day 2	Body Weight (gms) Day 7	Time of Death Due to Radiation (days)
3	900	BM IV	17.9	15.3	9[b]
4	900	BM IV	15.2	15.6	
5	900	BM IP	17.8	15.8	
6	900	BM IV	17.9	15.4	
7	900	none	19.4	17.6	11
8	900	none	16.3	13.5	9
10	900	none	18.8	14.5	10
11	900	none	13.5	-	4
1	600	BM IV	17.3	18.3	
2	600	BM IV	18.9	18.9	
3	600	BM IV	16.4	16.5	
4	600	none	22.5	22.2	
5	600	none	22.2	21.6	
6	600	none	16.1	15.3	

[a] BM = 2×10^7 syngeneic bone marrow cells

[b] Post-irradiation