



Unresponsiveness in Balb/c mice following repeated doses of sheep erythrocytes
by Philip Depoyster Thomson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Microbiology

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Abstract:

Balb/c mice were rendered unresponsive to sheep red blood cells by injections of 5×10^8 sheep red blood cells every other day from 7 days of age to adulthood. It was shown that unresponsiveness was specific by injection of burro red blood cells which produced normal plaque-forming cell responses in SRBC-unresponsive mice. Neither normal thymus nor bone marrow alone or in combination, nor normal peritoneal exudate cells could reconstitute unresponsive mice. However, SRBC-primed peritoneal exudate cells (80-90% macrophage cells) did reconstitute the plaque-forming response of unresponsive mice. Normal Balb/c mice were rendered unresponsive by injection of 32 hemagglutinin units of serum from unresponsive mice. Serum from unresponsive mice was absorbed with anti-IgG2. Serum absorbed in this manner failed to render mice unresponsive when administered passively. It was concluded that unresponsiveness to sheep red blood cells involves IgG2 which interacts with the macrophage and, in turn, inhibits antigen-macrophage interaction. Thus this system represents a feedback mechanism which prevents the presentation of antigen to the lymphocyte.

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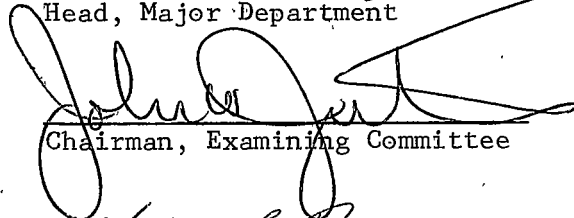
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ABSTRACT

Balb/c mice were rendered unresponsive to sheep red blood cells by injections of 5×10^8 sheep red blood cells every other day from 7 days of age to adulthood. It was shown that unresponsiveness was specific by injection of burro red blood cells which produced normal plaque-forming cell responses in SRBC-unresponsive mice. Neither normal thymus nor bone marrow alone or in combination, nor normal peritoneal exudate cells could reconstitute unresponsive mice. However, SRBC-primed peritoneal exudate cells (80-90% macrophage cells) did reconstitute the plaque-forming response of unresponsive mice. Normal Balb/c mice were rendered unresponsive by injection of 32 hemagglutinin units of serum from unresponsive mice. Serum from unresponsive mice was absorbed with anti-IgG₂. Serum absorbed in this manner failed to render mice unresponsive when administered passively. It was concluded that unresponsiveness to sheep red blood cells involves IgG₂ which interacts with the macrophage and, in turn, inhibits antigen-macrophage interaction. Thus this system represents a feedback mechanism which prevents the presentation of antigen to the lymphocyte.

INTRODUCTION

Burnet and Fenner (1949) predicted that embryonic exposure to an antigenic stimulus would lead to specific unresponsiveness or tolerance to that antigen in later life. This concept generated intensive research into the nature of the unresponsive state and the conditions required to induce it. The term tolerance was originally applied to the unresponsive state following the injection of living antigen, i.e., the injection of allogenic spleen cells into newborn mice. "Immunologic unresponsiveness", on the other hand, is a term originally suggested by Chase and used by Dixon to identify the group of phenomena whereby specific repression of the vertebrate immune response follows exposure to nonliving antigens (in Smith, 1961). Nonliving antigens as described by Smith (1961) include chemicals, proteins, carbohydrates, red blood cells, bacterial antigens, and similar complex mixtures.

It has generally been accepted by most that the terms "tolerance", "paralysis", "protein overloading", and "unresponsiveness" may represent aspects of the same basic process (Leskowitz, 1967).

Early studies illustrating the unresponsive condition include the feeding of cows milk, ox blood, and egg white to guinea pigs (Wells and Osborne, 1911), the injection of mice with pneumococcal polysaccharide (Felton and Ottinger, 1942), the vascular anastomosis of bovine twins by Owen (1945), and the inhibition of experimental drug

allergy by prior feeding of the sensitizing agent by Chase (1946).

More definitive studies were performed by Dixon and Maurer (1955) who, using rabbits given repeated injections of human plasma or bovine serum albumin (BSA) from shortly after birth until approximately 100 days of life, detected no BSA antibody, and they could detect no antibody for as long as 11 months after cessation of antigen infusion. The work of Smith et al. (1957) and Smith and Bridges (1958) shed more light on the mechanism of tolerance to protein antigens. These experiments showed that there was a relation between antigen dose and duration of tolerance, that persistence of the unresponsive state was dependent upon additional increments of antigen given while the animal was still tolerant, that tolerance is highly specific, that antigenically primed lymph node cells and spleen cells had no more reconstitutive effect than did normal lymph node cells and spleen cells when administered to tolerant animals, and that neither lymph node cells nor spleen cells from tolerant animals, when given to normal recipients, could elicit antibody production.

Smith (1961) proposed that in most circumstances none of the latter, productive phases of the immune response occur in the tolerant animal. Smith, however, agrees that there are other interpretations, e.g., that antibody is being produced constantly but bound to antigen immediately or that the antibody producing cell is

poised, but environmental conditions are not favorable for antibody production.

Since 1961, much work has been done on induction of the unresponsive state and on the cellular mechanisms involved. Table I shows some of the work done and is presented to show the diversity in experimental models. From these models, Dresser and Mitchison (1968) summarize the mechanisms involved in unresponsiveness. They state, "Immunologic paralysis is here defined as a central failure of responsiveness, brought about by exposure to antigen, in which immunologically competent cells (ICC) become unable to initiate synthesis of a restricted range of antibodies." For many antigen systems, the unresponsive condition follows an encounter of ICC with unprocessed antigen or massive doses of antigen which restrict the activity of potential antibody-producing cells. In some instances, paralysis or unresponsiveness was thought to be brought about by antibody blocking access of antigen to the receptors of the ICC. Regardless of the mechanism involved, unresponsiveness seems to depend on the recruitment of new competent cells by a thymus-dependent mechanism. Hence, in aged or in thymectomized animals, recruitment may occur slowly or not at all, and the precursoral antibody-forming cell that was affected in this mechanism was concluded to be a lymphocyte.

Table I. The diversity in experimental models of tolerance, 1962-1966.

Reference	Antigen; Animal; Assay system
Battisto and Miller 1962	BGG; guinea pig; delayed skin reaction
McGregor and Gowans 1963	BSA; mice; macrophage induction
Bathson, <u>et al.</u> 1963	Klebsiella polysaccharide; mice; antibody titer
Dietrich and Weigle 1963	SA and GG; mice; I ¹³¹ elimination
Claman and Talmage 1963	BGG; mice; prolongation of tolerance by thymectomy
Schwartz and Dameshek 1963	BSA; rabbit; agglutination, precipitation
Sercarz and Coons 1963	BSA; mice; Farr technique, agglutination
Gorman and Chandler 1964	hypothesis of the incompetent lymphocyte
Dorner and Uhr 1964	BSA; rabbit; I ¹³¹ elimination, agglutination
Friedman 1964	Shigella polysaccharide; mice, cell transfer
Mitchison 1964	BSA; mice; Farr technique, precipitation
Brooke 1965	Pneumococcus polysaccharide; mice; agglutination
Rowley and Fitch 1965	SRBC; rats; plaque-forming cells
Linscott and Weigle 1965	BSA; rabbit; Farr technique, precipitation
Nossal <u>et al.</u> 1965	Salmonella flagellin; rat; antibody titer, 2ME
Siskind and Howard 1966	Pseudomonas polysaccharide; mice; survivors
Austin and Nossal 1966	Salmonella flagellin; rat; antibody titer
Azar 1966	HGG; mice; immune elimination, IEP
Cruchaud 1966	BSA; rat; antibody titer, skin graft
Staples, <u>et al.</u> 1966	BGG; rat; irradiation of thymus and spleen
Weigle 1966	BSA; mice; agglutination, immune elimination
Thompson <u>et al.</u> 1966	Allogenic RBC; mice; I ¹³¹ elimination
Mitchell and Nossal 1966	Salmonella flagellin; rat; mechanism of tolerance

Chiller et al. (1971) have shown evidence that both thymus and bone marrow cells of adult mice can be made specifically unresponsive to human globulins, but that each cell population has its own special kinetics. Human IgG (HGG), as an antigen, shows slower induction of unresponsiveness in bone marrow than in thymus cells and is also needed in greater quantity in bone marrow. This work also shows, by means of reconstitution experiments, that only one cell type need be tolerant to render the animal unresponsive.

Nossal (1958) has shown that when suckling rats are injected repeatedly with sheep red blood cells (SRBC) for at least 7 weeks, they become specifically tolerant to SRBC. This phenomenon was thought to be due to antigenic material in the circulation which was presumed to react with and neutralize antibody molecules. This was shown not to be the case. Nossal repeated this work using mouse erythrocytes in rats and discovered that tolerance produced was more nearly complete and of longer duration, lasting up to 9 months.

Rowley and Fitch (1964) proposed a mechanism of tolerance in which rats made tolerant to SRBC were compared to rats made tolerant by passive immunization with homologous anti-SRBC serum. The plaque-forming response was reduced both in tolerant and passively immunized rats. Thus, the mechanism proposed was one of "feedback inhibition", in which anti-SRBC inhibited potential antibody-forming cells from

synthesizing antibody or prevented antigen from reaching and triggering the antibody producing cell (afferent branch).

McCullagh (1970), using rats made tolerant to SRBC, showed that the hemolysin response was enhanced in those rats which received thoracic duct lymphocytes incubated in vitro with primed peritoneal exudate cells. He envisioned some factor in the tolerant rat which is inimical to the initiation of an immune response in transfused, normal lymphocytes.

Miller and Mitchell (1970), working with adult CBA mice rendered tolerant to SRBC and maintained in the tolerant state with cyclophosphamide, have demonstrated that tolerance is not achieved at the non-thymus-derived lymphocyte level. Thymocytes also did not exhibit tolerance but, in contrast, thymus-derived cells of the thoracic duct did exhibit tolerance.

Feldman and Diener (1970) have shown suppression to SRBC to be mediated by preincubation of lymphoid cells with antibody in vitro, and have stated that this suppression is analogous to tolerance on the basis of antigen specificity and a central unresponsiveness.

The available evidence does not conclusively determine the precise nature of the unresponsive state to erythrocyte antigens nor does it definitely establish the role of various cellular elements in the induction of unresponsiveness. Thus, the purpose of this study was to

examine the state of specific unresponsiveness of mice to repeated doses of sheep erythrocytes and to determine the cell type and mechanism involved.

MATERIALS AND METHODS

Mice

Inbred Balb/c mice were used throughout this study. The mice were obtained from the National Institutes of Health (Bethesda, Maryland) in 1966 and were introduced into the conventionally-reared stock in 1967. The mice are currently maintained by brother-sister matings and fed autoclaved Purina 5010C and chlorinated, acidified water, ad libitum.

Immunologic Unresponsiveness

Mice were rendered immunologically unresponsive by intraperitoneal (I.P.) injections of 5×10^8 thrice-washed SRBC in a volume of 0.1 ml given every other day of life beginning at 7 days of age. Control mice were given 0.1 ml of sterile physiological saline in the same regimen.

The mice were assayed for responsiveness to the regimen of antigenation at day 10, at day 30, and at 30 day intervals. Blood was removed by retro-orbital bleeding and was pooled. Serum was collected from clots, which stood at room temperature for 1 hour, centrifuged at 4-10 C, and assayed for both hemagglutinin (HA) and hemolysin (HL) titer (Jutila, 1969). Mice were then sacrificed and spleens removed for assay in the slide modification of the Jerne plaque assay (Mishell and Dutton, 1967).

Irradiation

Mice were given 900 rad whole body gamma irradiation using a ^{60}Co source, delivering 128 rad/min onto a plexiglass cage (H and H plastics, Lincoln, Nebraska). This cage was placed in a 19 x 19 cm field on top of a turn table (Sieckman Construction Co., Lincoln, Nebraska), which was held 60 cm below the source. In this manner, 12 mice were irradiated at one time. Uniform irradiation was achieved by rotating the cage with mice at 2 rpm during irradiation. Maximum backscatter conditions were produced by placing a five-inch masonite block under the cage.

Reconstitution with Bone Marrow - Thymus

Bone marrow cells were obtained from the femur and tibial bones of unresponsive and normal mice by cutting off the epiphyses of bones free of all external tissue, and forcing the marrow from the marrow cavity by injecting 0.83% saline with a 26 gauge needle affixed to a 5 ml syringe. A single cell suspension was made by gently pipetting with a Pasteur pipette. The cells were washed in saline, larger particles allowed to settle out, and the cell suspension counted using a hemocytometer. Bone marrow cells were used in doses of 5×10^7 for reconstitution.

Thymus glands were removed from unresponsive and normal mice, and cells were prepared by gently screening through #80 mesh stainless

steel screens into 0.83% saline. The cells were washed once in saline and allowed to settle for 10 minutes. The cells were counted and a dose of 3.5×10^8 thymus cells were used for reconstitution. All manipulations involving cells were carried out at 4-10 C.

Mice were reconstituted with various combinations of normal and unresponsive thymus and bone marrow cells not more than 5 hours following irradiation. Thymus and bone marrow cells were mixed with 1×10^8 SRBC and 0.83% saline was used to bring the reconstituting dose to a volume of 0.5 ml. Cell suspensions were administered intravenously (I.V.) via the tail vein. After 8 days, the spleens were removed from recipients and cell assays were performed by a slide modification of the Jerne plaque assay (Mishell and Dutton, 1967).

Reconstitution with Peritoneal Exudate Cell

Nonimmune Balb/c mice were given 3 ml of fluid thioglycollate (Difco, Detroit, Michigan) by the I.P. route. Five days following thioglycollate, peritoneal exudate cells (PEC) were collected by washing the peritoneal cavity with 5 ml of Hanks minimal essential medium (MEM) (Microbiological Associates, Bethesda, Maryland) plus 5% fetal calf serum. In another experiment, SRBC were given intraperitoneally in a dose of 5×10^8 contained in 0.25 ml two hours prior to harvest. The peritoneal cells were washed once in MEM, counted and injected into unresponsive mice. PECs (5×10^7) were administered

intraperitoneally in 1 ml doses. Control mice received 5×10^7 unprimed peritoneal cells or received 5×10^8 SRBC alone. After 5 days, spleens from all mice were assayed by the modified plaque assay.

Immunoglobulin Quantitation

Unresponsive mice were selected at day 210 for immunoglobulin (Ig) quantitation. Mice were bled by retro-orbital puncture and serum was collected for assay in a modification of the single radial immunodiffusion technique of Masseyeff and Ziswiller (1969). Discs, 10 mm in diameter, were cut from 1.0% agarose (Sigma Chemical Co., St. Louis, Missouri) prepared in 0.2 M barbitol buffer, pH 8.6. Agar was poured into level, shallow trays rendering discs uniformly 1.5 mm in depth. Discs were placed at room temperature in an open, moist chamber for 1 hour. Antisera (Melpar, Falls Church, Virginia) specific for one of the Ig classes were diluted 1:20 in barbitol buffer and 0.05 ml was applied to each disc. The discs were then placed in a moist, covered chamber at room temperature for 12-18 hours.

Sera to be quantitated for immunoglobulins were pipetted into a 2 mm well cut into the center of each disc with a blunted 18 gauge hypodermic needle. Wells were then filled with 5 of antigen (serum) prepared in two-fold dilution steps from 1:128.

The precipitation reaction was allowed to progress for 48 hours at room temperature at which time excess protein was removed by

washing thrice with 0.83% saline. Discs were then stained with Acid Fuchsin for 30 minutes and destained with a mixture of water, methanol, and acetic acid (4:5:1).

The diameters of the precipitating rings were measured with the aid of a measuring microscope.

Passive "Turnoff"

Serum was collected from immunologically unresponsive mice by centrifuging blood taken by retro-orbital bleeding. The serum was titered and dilutions made with 0.83% saline to give an antiserum containing 32 HA units in each 0.1 ml. Test mice were given 32 HA units of serum, followed by 1×10^8 SRBC one hour later; both injections were administered I.P. Control mice were given 1×10^8 SRBC alone, or experienced no antigenation. All mice were sacrificed 5 days later, and their spleen cells were assayed for plaque-forming cells.

Immunoglobulin Class of Turnoff Antibody

Serum was collected from unresponsive mice and divided into 3 aliquots. One aliquot was absorbed with rabbit anti-mouse IgG₂ (Melpar, Falls Church, Virginia), one aliquot was absorbed with rabbit anti-mouse IgG (prepared by D. Manning, Montana State University, Bozeman, Montana), and one aliquot was not absorbed. All absorption

was done at 37 C. Normal mice received 32 HA units of one serum aliquot in 0.1 ml I.P. followed in 1 hour with 1×10^8 SRBC I.P. Control mice received 1×10^8 SRBC alone. Five days later, spleen cells were harvested and assayed for plaque-forming cells.

RESULTS

Unresponsiveness

The spleens of 110 mice receiving SRBC every other day from day 7 after birth were assayed for their plaque-forming cell (PFC) response in groups of 10 mice each beginning at day 10, again at day 30, and then at thirty day intervals thereafter. The results of these assays appear in Figure 1. A vigorous direct and indirect plaque response developed in treated mice by day 30 and peaked at two months of age. Thereafter, the response rapidly declined to less than 100 PFCs per 10^6 cells. Unresponsiveness to direct plaque assay was reached earlier (between 60 and 90 days) while unresponsiveness to indirect plaque assay was reached somewhat later (between 120 and 150 days). The unresponsive state continued to persist at day 300 and the immune response showed no signs of recovery 45 days after cessation of treatment with SRBC. That the unresponsive state is specific was shown when burro cells given to unresponsive mice (150 days after initiation of treatment) or to normal mice, produced a normal response (Table II).

The results of HA and HL titers appear in Figure 2. The kinetics of antibody synthesis in the interval day 10 to day 120 seem to mimic the PFC response. However, moderately high titers of antibody, presumably of the IgG class, were maintained in the unresponsive animal for some time after the plaque-forming response waned.

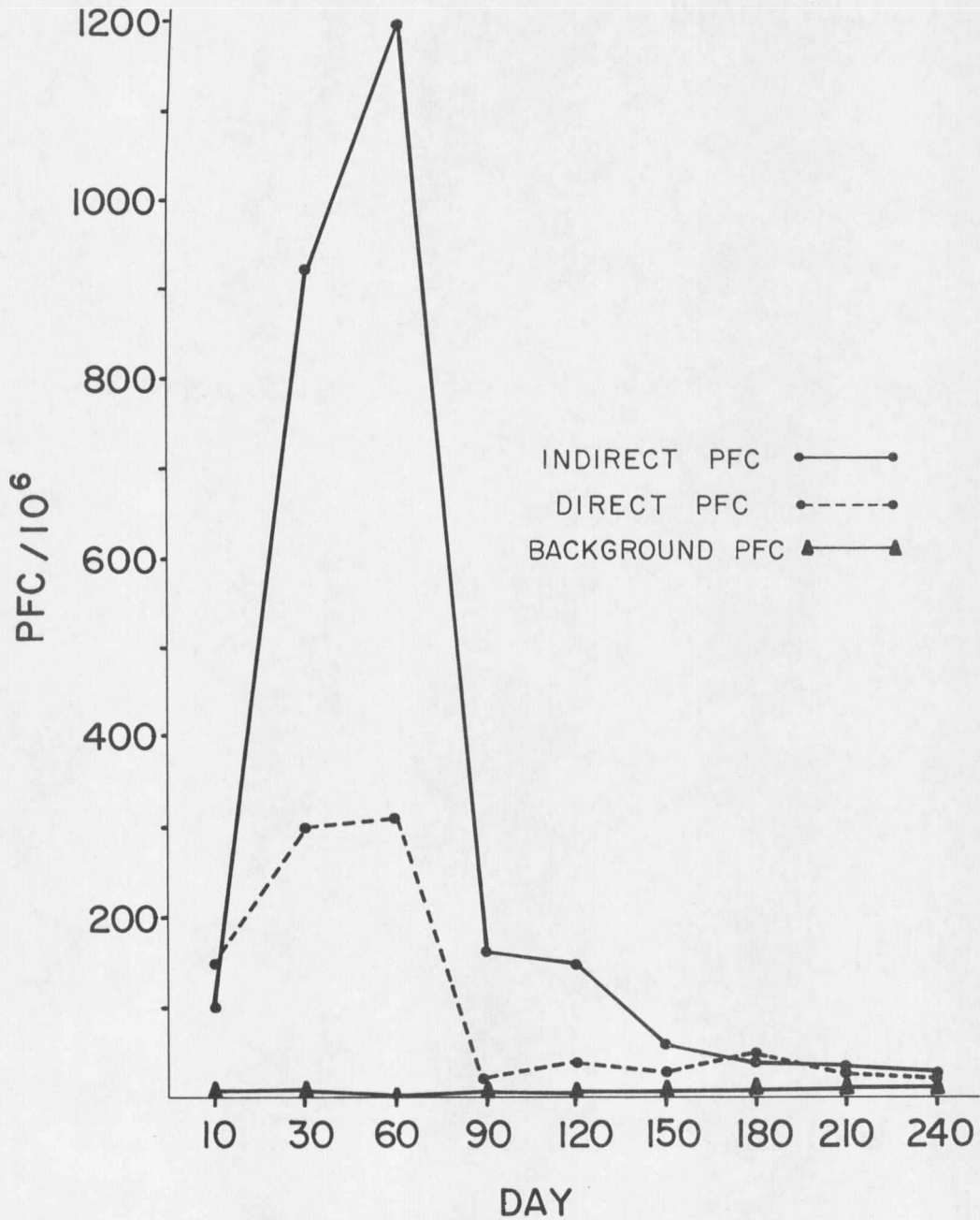


Figure 1. Plaque-forming cell (PFC) response in Balb/c mice treated with repeated doses of sheep erythrocytes.

Table II. Plaque forming cell (PFC) response of SRBC-unresponsive Balb/c mice to burro erythrocytes.

Treatment	PFC / 10 ⁶			
	Burro cells		Sheep cells	
	direct	indirect	direct	indirect
Unresponsive mice ^a given 5X10 ⁸ burro erythrocytes.	317	68	9	22
Normal mice given 5X10 ⁸ burro erythro- cytes.	541	82	2	0
Untreated mice	1	0	2	1

^a Mice assayed at day 150

