Immunosuppression induced in mice with specific antibody
by Joan Bader MacDonald

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

Abstract:
Immunosuppression by antibody was studied with particular emphasis on the role of the macrophage. Suppression was achieved by injecting 64 HA units of anti-SRBC serum. This is close to the threshold amount needed to establish suppression. The hemolysin and hemagglutinin titers of mice injected with anti-SRBC serum were 5% and 3% respectively of the mean control titers at day 10 post vaccination. Mice passively immunized with anti-SRBC serum responded normally to 5 x 10 7 human red blood cells but failed to respond to 5 x 107 SRBC. Sixty-four HA units was found to be sufficient to establish suppression and maintain suppression for 25 days at which time a slight increase in the response occurred. Balb/c mice receiving anti-SRBC serum 5 days previously were responsive to a second dose of 5 x 10 7 SRBC. Five days after receiving the second injection of sheep cells titers from experimental animals were 80% of the mean control titers. By day 25 post immunization there was no significant difference between the titers of experimental and control animals. Macrophages obtained from adult animals and incubated with anti-SRBC serum were not capable of inducing a response to SRBC from neonates. Macrophages which were incubated with normal mouse serum were effective in this regard.

Macrophages incubated with anti-SRBC serum were able to induce a response to guinea pig erythrocytes. Macrophages that were incubated with anti-SRBC serum, washed, and stained with fluorescein labelled rabbit anti-mouseγ globulin fluoresced brightly indicating a direct interaction between anti-SRBC serum and the macrophage.

It was concluded that immunosuppressive antibody exerts its effects on the macrophage and probably functions in vivo to bypass a second primary response once memory cells are established.

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at Montana State University, I agree that the Library shall make it freely available for inspection. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by my major professor, or, in his absence, by the Director of Libraries. It is understood that any copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Signature Joan Baker MacDonald

Date March 4, 1970

IMMUNOSUPPRESSION INDUCED IN MICE WITH SPECIFIC ANTIBODY

by 1141

JOAN BADER MAC DONALD

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

of

MASTER OF SCIENCE

in

Microbiology

Approved:

William G. Walter
Head, Major Department

John W. Tidd
Chairman, Examining Committee

F. Goering
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

March, 1970

ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to my advisor, Dr. John Jutila, for his patience, encouragement, and advice during the course of my studies.

I would also like to thank Mrs. Mary Ann Saunders for her assistance in typing the rough draft and Mrs. Darlene Harpster for typing the final manuscript.

I would also like to thank my husband, Paul MacDonald, for his encouragement and support of my graduate work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Immunosuppression by antibody was studied with particular emphasis on the role of the macrophage. Suppression was achieved by injecting 64 HA units of anti-SRBC serum. This is close to the threshold amount needed to establish suppression. The hemolysin and hemagglutinin titers of mice injected with anti-SRBC serum were 5% and 3% respectively of the mean control titers at day 10 post vaccination. Mice passively immunized with anti-SRBC serum responded normally to 5×10^7 human red blood cells but failed to respond to 5×10^7 SRBC. Sixty-four HA units was found to be sufficient to establish suppression and maintain suppression for 25 days at which time a slight increase in the response occurred. Balb/c mice receiving anti-SRBC serum 5 days previously were responsive to a second dose of 5×10^7 SRBC. Five days after receiving the second injection of sheep cells titers from experimental animals were 80% of the mean control titers. By day 25 post immunization there was no significant difference between the titers of experimental and control animals. Macrophages obtained from adult animals and incubated with anti-SRBC serum were not capable of inducing a response to SRBC from neonates. Macrophages which were incubated with normal mouse serum were effective in this regard.

Macrophages incubated with anti-SRBC serum were able to induce a response to guinea pig erythrocytes. Macrophages that were incubated with anti-SRBC serum, washed, and stained with fluorescein labelled rabbit anti-mouse γ globulin fluoresced brightly indicating a direct interaction between anti-SRBC serum and the macrophage. It was concluded that immunosuppressive antibody exerts its effects on the macrophage and probably functions *in vivo* to bypass a second primary response once memory cells are established.

INTRODUCTION

Inhibition of antibody synthesis by specific antibody has been studied extensively as a possible mechanism for regulating the concentration of specific antibody in the circulation. Theobald Smith (1909) first noticed that guinea pigs injected with diphtheria toxin-antitoxin mixtures containing excess antitoxin could not generate a response whereas guinea pigs injected with toxin-antitoxin mixtures at equivalence generated a strong response. Since then many authors have reported similar findings (Glenny and Sudmersen, 1921; Ramon and Zoeller, 1933; Barr et al., 1950).

Specificity is the most striking characteristic of this type of immunosuppression. Using the diphtheria toxin-antitoxin system, Uhr and Baumann (1961) suggested that suppression was specific after showing that non specific γ globulin did not suppress antitoxin formation. Rowley and Fitch (1964) demonstrated that rats suppressed with anti-sheep erythrocyte serum (SRBC), while not able to respond to SRBC, were fully responsive to purified Salmonella typhosa flagella. Likewise, rats receiving anti-S. typhosa did not respond to S. typhosa antigen but did respond to SRBC. Similarly, Axelrad and Rowley (1968) demonstrated that animals treated with anti-SRBC did not respond to SRBC but did elicit a response to horse red blood cells and Bordetella pertussis vaccine.

On the basis of this specificity it has been speculated that the mechanism for suppression is antigen elimination or masking of the antigenic site (Britton and Moller, 1968; Dixon, Jacot-Guillarmod, and McConahey, 1967; Uhr and Moller, 1968). Uhr and Finkelstein (1963) found that below a critical level of antigen the rate of antibody formation is antigen dependent. This lends support to the notion that the retention and level of antigen might act as a control mechanism.

Further support for the concept of antigen elimination was given by differences found in the suppressive capabilities of 19S and 7S antibodies. Moller and Wigzell (1965) determined that 7S antibody was 115 times more efficient at suppression than 19S antibody. Finkelstein and Uhr (1964) also observed that 19S antibody was a less effective suppressant than 7S antibody. This phenomenon might be explained by γ M's short half life and γ G's greater affinity for antigen. This argument was supported when it was shown that high avidity antibody was an effective suppressant at concentrations much lower than those needed when using low avidity antibody (Walker and Siskind, 1967).

The use of antibody fragments has also supplied valuable information for if the mechanism of suppression is antigen elimination, $F(ab')_2$ should be as effective as whole antibody. However, if

antibody is exerting its effect directly on cells, removing the Fc fragment should impair the antibody's suppressive abilities. The initial work with antibody fragments indicated the $F(ab')_2$ fragment was as effective as whole antibody in achieving suppression (Cerretoni, McConahey, and Dixon, 1969). However, St. C. Sinclair (1969) reported that whole antibody was from 100 - 1000 times more effective than $F(ab')_2$. This difference might be explained by the short half life of antibody fragments. Investigating this possibility, St. C. Sinclair, Less, and Elliott (1968) used complexes of $F(ab')_2$ and its corresponding antigen which have longer half lives than unbound $F(ab')_2$. Their results again showed that whole antibody is far more effective than antibody fragments. These investigators feel that the conflicting data of other authors is due to the use of preparations contaminated with whole 7S antibody.

The amount of antibody necessary for suppression is also important. One may postulate that there must be enough antibody present to cover all or most antigenic determinants. Dixon and coworkers recently produced evidence for this concept using Keyhole Limpet Hemocyanin (KLH) as the antigen (Dixon, Jacot-Guillarmod, McConahey, 1967). However, using the classical diphtheria toxin-antitoxin system, Uhr and Baumann (1961) achieved suppression with fewer antibody molecules than the minimum number of known major

determinants on the antigen. Haughton and Nash (1969) did the first truly quantitative study by isotopically determining that 3-4 x 10^{14} antibody molecules were required to saturate all of the antigenic determinants on 5 x 10^{8} SRBC. However suppression was achieved with as little as 8 x 10^{11} antibody molecules.

Inconclusive evidence for antigen elimination led to studies on the effects of passive antibody on cells. Rowley and Fitch (1964) investigated the immunocompetence of passively immunized lymphocytes. They transferred 5 x 10^{8} passively immunized spleen cells into an x-irradiated host which was then challenged with antigen. In all cases titers in animals receiving passively immunized spleen cells were lower than those found in animals receiving normal spleen cells. However, this work could not be repeated by other investigators (Moller, 1964; Uhr and Moller, 1968) and it was postulated that Rowely and Fitch passively transferred antibody with their cells.

Greenburg and Uhr (1968) investigated the incorporation of H^{3} leucine in lymph node cells from immune animals and lymph node cells treated with antibody 48 hours after immunization. Immune cells not treated with antibody showed a linear increase in leucine incorporation from day 5 to day 7 while immune cells exposed to antibody showed a marked increase from day 3 to day 4 followed by a

decline to day 7. The significance of this altered protein synthesis has not been fully investigated.

Pierce (1969) studied the effect of passive antibodies on cells using a tissue culture system. Lymphoid cells and macrophages in tissue culture together produced antibody when exposed to antigen. If the culture was incubated with specific antibody before exposure to the antigen no antibody was produced. When lymphoid cells were incubated with antibody, washed, mixed with macrophages and exposed to the antigen, normal levels of antibody were produced. However, when macrophages were incubated with antibody, washed, mixed with lymphocytes and exposed to antigen, no antibody was produced.

This work was given further support by the studies of Ryder and Schwartz (1969). They injected SRBC into passively immunized mice where they were presumed to be coated with antibody. It was found that such cells were fully antigenic, whereas cells left in the original recipients did not stimulate a response. In other experiments they showed that SRBC fed macrophages from passively immunized mice failed to elicit a response whereas mice receiving SRBC laden macrophages from untreated mice responded.

The purpose of this study was to further examine the effects of passive antibody paying particular attention to the macrophage and the possible interactions between the macrophage and passively

administered antibody.

MATERIALS AND METHODS

Mice

Adult inbred Balb/c and randomly bred Swiss Manor mice 3 to 6 months of age were used throughout the study. The former were obtained in 1966 from the National Cancer Institute (Bethesda, Maryland) in the germ free state and were conventionalized six months later. The Swiss Manor mice were obtained in 1964 from Manor Farms (Straatsburg, New York). The mice are maintained by brother-sister matings. All mice were fed Purina Laboratory Chow and water *ab libitum*.

Preparation of Antisera

Ten animals were given 3 intraperitoneal (i.p.) injections of 0.1 ml thrice-washed 10% SRBC at 7 day intervals. Five days after the final injection the animals were bled from the retro-orbital plexus and the blood was pooled. Serum was separated on a sucrose density gradient by ultracentrifugation at 37,000 RPM in a SW-39 rotor for 17 hours using a Beckman Model L-2 Ultracentrifuge (Beckman Instruments Inc., Palo Alto, California). A gradient consisting of 1.2 ml of 40%, 30%, 20% and .7 ml of 10% sucrose dissolved in .01 M phosphate buffered saline (PBS) was prepared in tubes. Ten fractions were collected dropwise from the bottom of the tubes and titered. Fractions containing high concentrations of 7S

antibody were pooled, dialyzed against PBS, and titered again. The antibody, diluted to contain 64 hemagglutination units per 0.1 ml, was stored frozen in 0.5 ml aliquots.

Determination of Antibody Titers

Hemagglutinin titers were determined by serial two-fold dilutions in PBS. To a 0.1 ml volume of diluted serum, 0.1 ml of 1% SRBC was added. The tubes were incubated in a 37°C water bath for 30 minutes. The tubes were centrifuged at 3,700 RPM for 1 minute, shaken, and the degree of agglutination scored as a 4+ (pellet rises in one clump), 3+ (pellet rises in several large clumps), 2+ (pellet rises in several small clumps and loose cells), 1+ (pellet rises in loose cells with a few persistant clumps), or negative (pellet rises in a cloud of loose cells). The last dilution giving a score of 1+ or more was taken as the titer.

The hemolysin titer was determined by adding .05 ml guinea pig complement (BBL) diluted 1:5 to the above agglutination system. Tubes were incubated for 30 minutes at 37°C and refrigerated overnight. The dilution of the last tube showing complete hemolysis was taken as the titer.

Immunosuppression

The mice were injected i.p. with 64 hemagglutination (HA) units of 7S anti-SRBC antibody contained in 0.1 ml. Controls received 0.1 ml PBS i.p. One hour later all mice received 5×10^7 SRBC. To determine the duration of suppression mice were bled and their sera titered on days 3, 5, 10, 17, 24, 32, and 42. In other experiments 5×10^7 human red cells were injected to check for specificity. Controls received saline and human red blood cells. On days 5 and 10 the mice were bled from the retro-orbital sinus and their sera titered. To determine whether suppressed mice were capable of yielding a secondary response the mice were given a second dose of 5×10^7 SRBC several days after immunosuppression. Animals were bled and their sera titered on days 7, 17, 25, and 30.

Cell Transfer into Neonates

Mice from 1 to 3 months old were injected i.p. with 3 mls of thioglycollate (Difco). Three to seven days later the mice were sacrificed and their peritoneal covities washed twice with 5 mls. Hanks Balanced Salt Solution (Microbiological Associates Inc., Bethesda, Md.) containing 1% fetal calf serum and 10 μ heparin/ml. Cell preparations were divided into experimental and control groups. The experimental group was incubated with 64 HA units anti-SRBC per

donor mouse for one hour at 37°C, while the control group was simultaneously incubated with an equal volume of normal mouse serum. Ten x 10^6 cells were injected i.p. into neonates according to the technique of Argyris (1968). Three days after the injection of peritoneal cells, 5 x 10^7 SRBC were injected i.p. into the mice. Four days later mice were sacrificed, their spleens removed and assayed using the plaque technique of Jerne and Nordin (1963).

Fluorescent Antibody

Fluorescein labelled rabbit anti-mouse γ globulin (Difco) was used to examine macrophages from passively immunized mice. The antisera was purified by passage through a column of DEAE-Cellulose according to the technique of Wood, Thompson, and Goldstein (1965). Fractions were assayed for fluorescent labelled antibody by measuring the absorption at 280 mμ and the %T at 485 mμ on a Coleman 124 Double Beam Spectrophotometer (Coleman Instruments Division, Maywood, Illinois).

Peritoneal exudates were induced in mice with 3 mls thioglycollate broth. Macrophages were removed as above and incubated at 37°C for 1 hour with 0.1 ml 1:100 rabbit anti-mouse γ globulin to bind any non-specific globulins that might be on the macrophages. The preparations were washed twice and incubated with 128 HA units

anti-SRBC or normal mouse serum (NMS). The macrophages were washed twice, smeared on clean slides, air dried, and fixed in 95% ethanol. Smears were stained with fluorescent rabbit anti-mouse γ globulin for two hours in a moist atmosphere, washed in PBS for two twenty minute intervals, and mounted with phosphate buffered glycerin. Slides were observed using a Leitz Labolux fluorescent microscope with BG-12 and BG-38 filters.

RESULTS

Degree of Immunosuppression Achieved Using Passive Antibody

Balb/c and Swiss Manor mice suppressed by anti-sheep erythrocyte (SRBC) serum were bled and their sera titered on days 3, 5, and 10 post vaccination. The results of hemolysis and hemagglutinin titrations are shown in Tables I and II.

The hemolysin response in Balb/c mice was completely suppressed at day 3, 11% of mean control titers at day 5, and 5% of mean control titers at day 10 post vaccination. Moreover, all of the control mice responded to immunization while only 57% of experimental animals responded.

Similar results were obtained for the hemagglutinin response. The anti-SRBC response of Balb/c mice treated with anti-SRBC antibody was totally suppressed at day 3, 1% of mean control titers at day 5, and 3% of mean control titers at day 10. Once again all of the control mice responded while only 33% of the experimental mice responded. The results obtained in Swiss Manor mice (Table II) were virtually identical to those described above and eliminate, in effect, the possibility of strain effect.

Specificity of Immunosuppression

Balb/c and Swiss Manor mice given anti-SRBC responded normally to 5×10^7 human red blood cells but failed to respond to 5×10^7

Table I

The effect of passive immunization on the hemolysin and hemagglutinin response in Balb/c mice.

Treatment[a]	Days of Titration[b]	Antibody Titers[c]					
		Hemolysin			Hemagglutinin		
		No. responding[d]	Mean	Range	No. responding	Mean	Range
Anti-SRBC	3	0/9	0	0	0/9	0	0
	5	4/7	3.3	0-8	1/7	.14	0-2
	10	4/7	1.6	2-4	2/7	.3	0-2
Saline Control	3	3/3	3.3	2-4	0/3	0	0
	5	3/3	16	8-32	3/3	11.3	4-16
	10	2/2	20	8-32	2/2	10	4-16

[a] The mice received a single i.p. injection of Balb/c anti-SRBC 1 hr before receiving 5×10^7 sheep erythrocytes, i.p.

[b] Day after immunization mice were bled for antibody titrations.

[c] Titers are expressed as reciprocals of serum dilutions.

[d] The numerator indicates the number of mice responding: the denominator the total number immunized.