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B CELL KILLING WITH ANTI-Ig SERA PLUS C'

REQUIREMENTS FOR ROUTINELY KILLING B CELLS WITH ANTI-IMMUNOGLOBULIN SERA PLUS COMPLEMENT *

*This research supported by grants from the National Institute of General Medical Science (Nos. GM-20832 and GM-17367).

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INTRODUCTION

In general, cells with antibody bound to surface constituents are readily lysed in the presence of complement. Large amounts of anti-immunoglobulin antibody (anti-Ig) can bind to the surface immunoglobulin on mouse B cells (bone marrow-derived lymphocytes). Yet, with most anti-Ig sera these cells appear to be resistant to lysis treatment with anti-Ig plus complement. Thus, despite prolonged efforts by many investigators, no reliable cytotoxicity method has been developed for using anti-Ig to deplete B cells from mouse spleen and lymph node preparations.

In this publication we will show that B cells are routinely lysed by anti-Ig plus complement, provided that a reversible metabolic inhibitor such as sodium azide is present during the incubation with anti-Ig. The conditions for anti-Ig, plus complement, to be cytotoxic for B cells are the same as those required for the inhibition of cap formation of the surface immunoglobulin. Allowing cap formation but inhibiting removal of the surface immunoglobulin leads to non-cytotoxic conditions.

MATERIALS AND METHODS

Cytotoxic Assay. ^{51}Chromium cytotoxic testing, as described by Wigzell (1) and modified by Bomford et al. (2) was used. Briefly, splenic lymphocytes from adult C3H.SW mice were labeled with $Na_2{}^{51}CrO_4$. The cells were washed in Balanced Salt Solution (BSS) (3) containing 0.1% bovine serum albumin (BSA) with or without metabolic poisons (e.g., sodium azide, dinitrophenol) as stated in the Results Section. An equal volume of cells (5×10^6/ml) was added to 0.1 ml of serial dilutions of rabbit anti-mouse immunoglobulin (RAMIG). The cells were incubated at room temperature for 30 minutes, washed and resuspended in 0.2 ml normal rabbit serum (NRS) (as a source of complement), diluted 1:10 with BSS containing 0.2% sodium azide and 0.1% BSA, then incubated 30 minutes at 37°C. Cold BSS (1 ml) was added to each sample, then the tubes were centrifuged and the supernate was decanted into counting vials and counted in a gamma scintillation counter. Maximum counts released was determined by freezing and then thawing, three times, aliquots containing 5×10^5 labeled cells. The percentage of ^{51}Cr release was calculated.

Rabbit Anti-Mouse Immunoglobulin Antisera. Rabbits were immunized with either 100 μg of DEAE purified normal mouse immunoglobulin or 250 μg of a cocktail of purified mouse myeloma proteins (MOPC-21 = γG_1, GPC-8 = γG_{2a}, MPC-31 = γG_{2b}, MOPC-104 = γM, MPC-1 = γA) in complete Freund's adjuvant. The rabbits were boosted 3-4 weeks later with 100 μg of the same immunogens with which they had been primed, and bled 7-10 days later. The sera were harvested, heat-inactivated at 50°C for 30 min, adsorbed with 10% volume of mouse thymocytes, in the cold, for 30 min., and stored at -20°C.

Mitogen Response. Spleen cell suspensions (5×10^6/ml) were treated with an equal volume of 1:50 dilution of RAMIG, washed, resuspended in an equal volume of NRS diluted 1:10 with BSS containing 0.2% sodium azide and 0.1% BSA and incubated at 37°C for 30 min. The cells were then washed and resuspended in RPMI-1640 (H-18, powder, Grand Island Biological Co., Grand Island, New York) supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg /ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES buffer (No. 130440, GIBCO) to 4×10^6 cells/ml, without recounting. The cells were dispersed into microtitre trays (#3040, Becton Dickinson and Co., Falcon Division, Cockeysville, Maryland), 4×10^5 cells/well, and cultured at 37°C with and without phytohemagglutinin (PHA) (HA16, purified, Burroughs Wellcome Co., Greenville, North Carolina), concanavalin A (Con A, Cat. No. 234567, Grade A, Calbiochem, La Jolla, California), or Escherichia coli 011:B4 lipopolysaccharide-W (LPS, #3122-25, Difco Laboratories, Detroit, Michigan) over a wide dose range. Only the responses at the optimal dose, for each mitogen are reported in the Results Section. After approximately 65 hours incubation, the cultures were pulsed with

1 μCi of ^{3}H-thymidine ((methyl-^{3}H)-thymidine, 6 ci/mol, Cat. No. 253396, Schwarz/Mann, Division of Becton, Dickinson and Company, Ornageburg, New York). The cultures were harvested 8 hours later using a multiple automated sample harvester (MASH-II, Microbiological Associates, Bethesda, Maryland). The samples were counted in a tri-carb liquid scintillation spectrometer (Packard Instruments Co., Inc., Downers Grove, Illinois).

RESULTS

Specificity of RAMIG Cytotoxicity for B Cells

Approximately 50% of mouse spleen cells can routinely be lysed with rabbit anti-mouse immunoglobulin (RAMIG) serum and complement, provided that 0.2% sodium azide is present during treatment. As data presented in Table I show, B cells are selectively killed by this treatment and the residual viable population is correspondingly enriched for T cells (4).

Mouse spleen cells were incubated for 30 minutes at room temeprature with RAMIG, at a 1:50 dilution, in the presence and absence of 0.2% sodium azide. The cells were washed and both groups incubated with rabbit complement containing 0.2% sodium azide at 37^{o}C for 30 minutes. The cells were washed, then one-half of each group incubated with anti-Thy-1.2, washed and stained with fluorescein labeled rabbit anti-mouse IgG (adsorbed with mouse Fab) to stain residual T cells. The other half was stained with fluorescent labeled goat anti-rabbit immunoglobulin to stain residual B cells. Percentages of T and B cells were then estimated by fluorescence microscopy. The spleen cells treated with RAMIG and complement showed roughly a 2-fold increase in the number of Thy-1 positive (T) cells. Conversely, there was roughly a 20-fold decrease in the number of immunoglobulin positive (B) cells (Table I). The cytotoxic activity of the RAMIG was completely removed by passage over a Sepharose 4B column conjugated with normal mouse immunoglobulin (5), thus showing that all of the cytotoxic activity was due to anti-immunoglobulin.

Functional Studies of Cells Surviving Treatment with RAMIG Plus Complement

Functional evidence that B cells and not T cells are lysed by such treatment is obtained with the mitogen studies. The PHA and Con A responses, which have been shown to be T cell responses (6), are unimpaired. On the other hand, the LPS response, which has been shown to be a B cell response (7), is drastically reduced. Mouse spleen cells were incubated with RAMIG, at a dilution of 1:50 for 30 minutes at room temperature. The cells were washed and incubated with rabbit complement containing 0.2% sodium azide for 30 minutes at 37^{o}C. The cells were washed and tested for mitogen reactivity. The results of the optimal response for each mitogen are shown in Fig. 2. Treatment with RAMIG plus complement, in the presence of 0.2% sodium azide, eliminated the response to LPS without reducing the response to PHA or Con A.

Requirement for Sodium Azide

When tested in the presence of 0.2% sodium azide, all of the rabbit anti-mouse immunoglobulin (RAMIG) antisera tested were cytotoxic for approximately 50% of mouse spleen cells. The antisera differed only in their titer, ranging from 1:32 to 1:512 (Table II). On the other hand, no cytotoxicity was detectable when spleen cells were treated with RAMIG in the absence of sodium azide. The percentage of T and B cells (Table I) and the response to both T and B cell mitogens remained the same as non-treated controls (Fig. 1)

Sutdies of surface Ig after RAMIG treatment indicate that the lack of cytotoxicity in the absence of sodium azide is not due to

antibody induced removal of surface immunoglobulin via pinocytosis or exocytosis. Under the conditions used here for RAMIG treatment, Ig remains on the cell surface whether azide is present or not. The only difference we could detect between cells treated with or without azide present is that in the presence of azide the surface Ig remains randomly distributed whereas in the absence of azide the Ig forms caps.

For these studies, mouse spleen cells were incubated with RAMIG (first step) in the presence or absence of sodium azide and then stained with fluorescein labeled goat anti-rabbit immunoglobulin (second step) to allow quantitation and visual location of the bound RAMIG. The second step was carried out in both cases in the presence of sodium azide to prevent further redistribution of RAMIG. The stained cells were analyzed on the fluorescence-activated cell sorter (FACS) (8) and by fluorescence microscopy.

FACS analysis (Fig. 2) shows there is essentially no difference in the number of fluorescent labeled cells. There was slightly less surface immunoglobulin, as measured by fluorescence intensity, when the cells were treated in the presence of azide as compared to treatment in the absence of azide.

Visual examination revealed a difference in the distribution of the Ig molecules on the cell surface. The cells treated with RAMIG in the presence of sodium azide showed ring or diffuse staining. The cells treated with RAMIG in the absence of azide, however, showed staining at one pole ("capped" stain).

DISCUSSION

We have shown here that splenic B cells are specifically lysed by treatment with RAMIG plus complement. Killing, however, is dependent on the presence of azide or other metabolic inhibitor which prevents antibody-induced capping. Thus, rabbit anti-mouse Ig, on the surface of a B lymphocyte, will lyse the cell, upon the addition of complement, if it is not redistributed into a cap. On the other hand, if the rabbit anti-mouse Ig is redistributed into a cap, it will not lyse the cell upon the addition of complement.

The results offer some insight into the mechanism of antigenic modulation (9). Boyse et al., have shown that when cells are exposed to antibody directed against certain cell surface constituents, *in vivo* or *in vitro*, they rapidly lose their sensitivity to cytotoxicity upon re-exposure to antisera in the presence of complement. Raff and De Petris (10) have suggested that antigenic modulation is due in part at least to antibody induced pinotytosis, i.e., that antibody removes antigen from the cell surface. Our results suggest that capping alone, could account for antigenic modulation. Thus, when antibody is added to cells with diffusely distributed surface antigenic determinants, the antibody induces cap formation. Once in the cap, the antibodies are no longer lytic when complement is added and most or all of the antigen sequestered. This leaves insufficient quantities of antigen on the cell surface to bind more antibody. Thus the cell becomes non-susceptable to attack on re-exposure to antiserum and complement.

A similar explanation can be offered for the "lyso-strip" method (11,12) for rendering cells non-susceptable to complement dependent

lysis with one antiserum but susceptable with another. In the first the cells are incubated with antiserum and the corresponding antigenic sites are coated. Next a second layer of antibodies is added by incubating the cells with anti-immunoglobulin allowing capping of the antigenic sites. Finally, the cells are tested in standard cytotoxic assays. The cells are now resistant to lysis when additional antibodies of the same specificity and complement are added. On the other hand, antigens not present on the redistributed antigens should be randomly distributed on the cell surface and are lysed when exposed to antibodies against these antigens in the presence of complement. The obvious explanation is that the first or second antiserum does not have to be removed (stripped) from the cell surface. Capping of the first antigen is alone sufficient to render the cell resistant to complement-dependent lysis by the first antiserum.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Leonore A. Herzenberg for her critical reading of this paper.

TABLE I

REMOVAL OF Ig-BEARING (B) CELLS AND NOT THY-1-POSITIVE (T) CELLS BY TREATMENT WITH RAMIG AND COMPLEMENT WITH AZIDE

Treatment	1st Step	2nd Step	Stained (%)
RAMIG without sodium azide, plus complement	--	GAMIgG*	9.6
"	Anti-Thy-1.2	GAMIgG	46.7
"	--	GARIgG†	37.9
RAMIG with 0.2% sodium azide, plus complement	--	GAMIgG	2.9
"	Anti-Thy-1.2	GAMIgG	82.2
"	--	GARIgG	2.7

*Fluorescein labeled goat anti-mouse IgG immunoglobulin

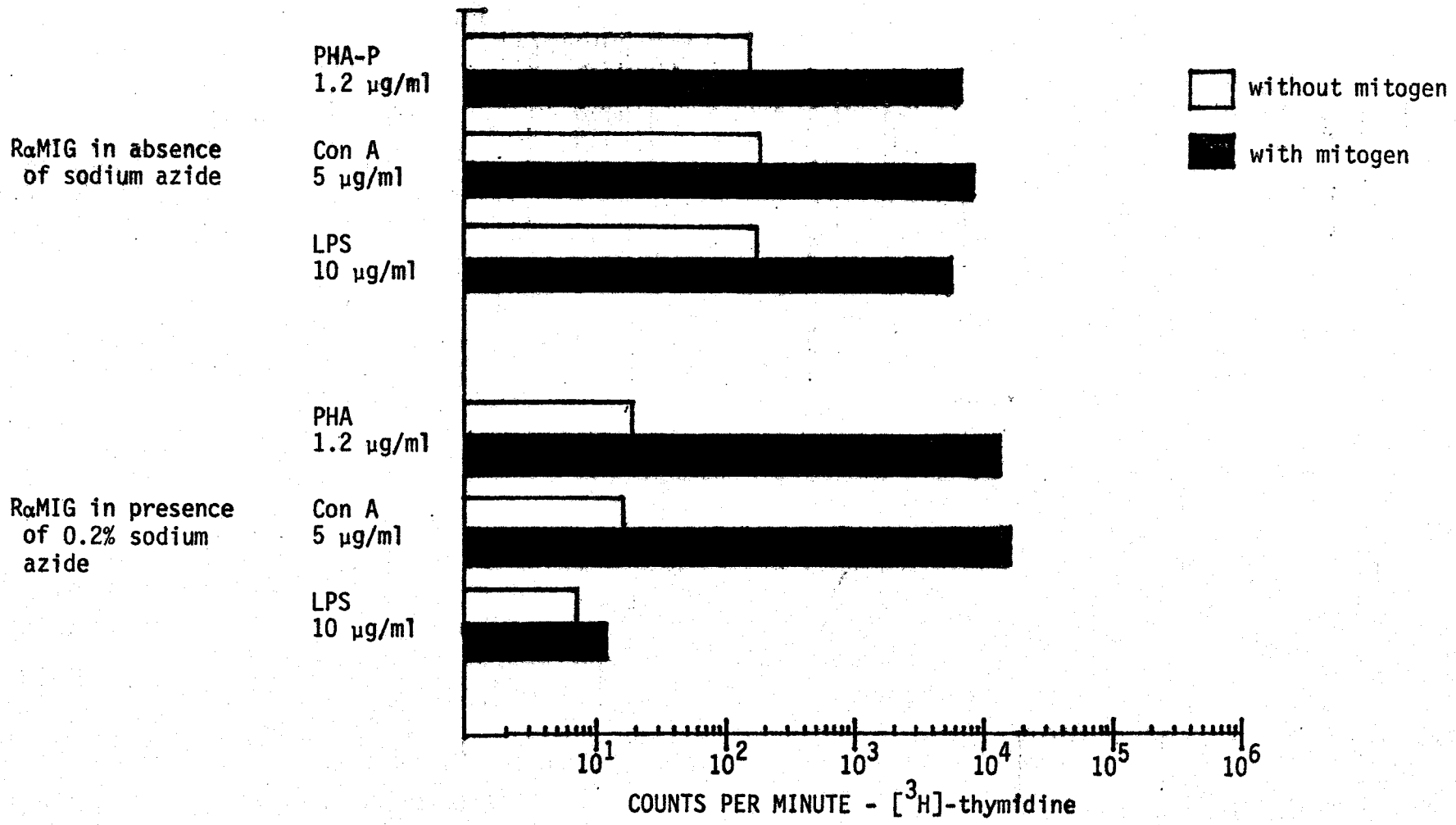
†Fluorescein labeled goat anti-rabbit immunoglobulin

TABLE II

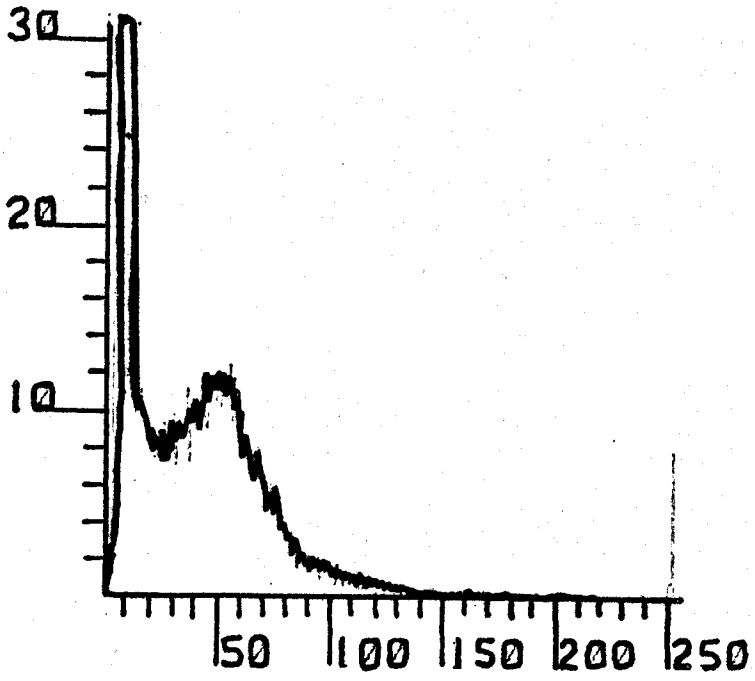
CYTOTOXIC TITER OF INDIVIDUAL
RABBIT ANTI-MOUSE IMMUNOGLOBULIN

Rabbit No.	Titer*
1	1:32
2	1:64
3	1:64
4	1:32
5	1:64
6	1:128
7	1:128
8	1:128
9	1:64
10	1:512

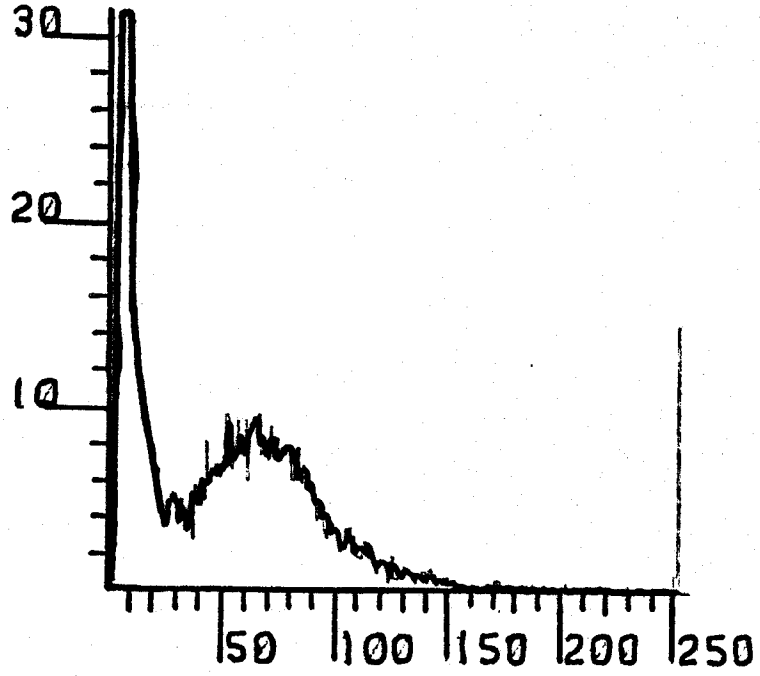
*Dilution of antiserum that lyses 50% of mouse spleen cells


RαMIG in absence of sodium azide
PHA-P 1.2 µg/ml
Con A 5 µg/ml
LPS 10 µg/ml
RαMIG in presence of 0.2% sodium azide
PHA 1.2 µg/ml
Con A 5 µg/ml
LPS 10 µg/ml
without mitogen
with mitogen
10^1
10^2
10^3
10^4
10^5
10^6
COUNTS PER MINUTE - [^{3}H]-thymidine

a)



b)



RELATIVE NUMBER OF CELLS

FLUORESCENCE INTENSITY

LEGENDS FOR FIGURES

Fig. 1. Response to the Mitogens PHA, Con A and LPS After Treatment With RAMIG Plus Complement.

Spleen cells were incubated with a 1:50 dilution of RAMIG, in the presence or absence of 0.2% sodium azide, for 30 minutes at room temperature. The cells were washed, incubated in normal rabbit serum, diluted 1:10 with BSS containing 0.2% sodium azide, for 30 minutes at 37°C. The cells were washed and cultured for 3 days with and without mitogen. The cultures were pulsed with (^{3}H)-thymidine for 8 hours, harvested and the amount of radioactivity determined.

Fig. 2. Fluorescence Staining for Surface Immunoglobulin.

Spleen cells were incubated at room temperature for 30 minutes with a 1:50 dilution of RAMIG in the presence (a) or absence (b) of 0.2% sodium azide. Both groups were washed, resuspended in BSS containing 0.2% sodium azide and 0.1% BSA, and incubated for 30 minutes at 37°C. Both groups were then stained with fluorescein-labeled GARIG and analyzed on the FACS.

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