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The Role of Mitogens and Antigens in the Generation of Antibody-Producing Human B Lymphocytes

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Satisfactory experimental systems with which to study the antigen specific humoral immune response of human peripheral blood lymphocytes have not been available until recently. A commonly used method for the study of antibody production by human lymphocytes is that developed by Fauci and Pratt. This system is considered to be antigen nonspecific since the antigen against which the determined antibody is directed is not added to the cultures. We show here that the assumption of the Fauci-Pratt system being antigen nonspecific is not justified. An essential ingredient of this culture system is human serum that has been exhaustively absorbed with antigen (sheep red blood cells). This absorption procedure causes shedding of highly immunogenic antigenic fragments whose immunogenic activity we demonstrated using a recently developed antigen-dependent culture system. In the latter system, we have shown that the control of suppressor cells is a critical factor for the successful induction of antibody responses, particularly in view of the fact that lymphocyte mitogens must be added to cultured human lymphocytes to support their responsiveness. Appropriate timing of mitogen addition to the cultures was found to be an effective means of preferentially stimulating helper or suppressor activity. Pokeweed mitogen, a mitogen known to act on B and on T lymphocytes, stimulates B-cell responses readily but abrogates them prematurely by the simultaneous activation of suppressor cells. When pokeweed mitogen is added to an ongoing response with a delay of 48 hr, it enhances antibody responses markedly, presumably by providing additional help to B cells at a time when they have lost susceptibility to suppressor-cell effects.

KEY WORDS: B lymphocytes; antibody production; suppressor cells.

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INTRODUCTION

Pokeweed mitogen (PWM) has been commonly used to stimulate human peripheral blood B cells to produce immunoglobulins *in vitro*. Ig-producing capacity has been determined by measuring the production of intracellular Ig (1-3) or Ig-secreting cells (4). Fauci and Pratt (5, 6) described a culture system in which PWM-stimulated peripheral blood B lymphocytes generate anti-SRBC antibody-producing cells. SRBC were chosen as targets with the contention that among the antibodies secreted, those that would react with this antigen could be detected in a hemolytic plaque assay. This system was not considered to be antigen driven since antigen (SRBC) was not added to the cultures.

Human serum is an indispensible ingredient of the Fauci-Pratt culture system. Absorption of human serum with SRBC was found to be necessary for anti-SRBC PFC responses to occur. The requirement for serum absorption was explained with the removal of anti-SRBC antibody which would interfere with anti-SRBC PFC responses. Alternatively, however, it is possible that SRBC shed antigenic material into the serum during the absorption procedure. This possibility is tested in this paper. We have used for this purpose a recently developed method for antigen-specific production of antibody *in vitro* (7). Essential ingredients of this culture system are human serum and heat-inactivated *Staphylococcus aureus* bacteria (Cowan strain) in addition to antigen. We show here that SRBC-absorbed human serum contains highly immunogenic SRBC antigens. The relevance of this finding for the generation of antibody-forming cells with the help of PWM is investigated in view of the immune-enhancing and immune-suppressive activities of PWM.

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METHODS

Isolation of Lymphocytes. Peripheral blood mononuclear cells (PBM) were obtained from heparinized venous blood of healthy volunteers by flotation on standard Ficoll-Hypaque gradients. T- and non-T-cell preparations were obtained by rosette formation of T lymphocytes with neuraminidase-treated SRBC and subsequent flotation on Ficoll-Hypaque gradients (8). Non-T-cell preparations were further partially depleted of adherent cells by incubation at 37° C in plastic petri dishes for 30 min in medium containing 20% FCS. Nonadherent cells were decanted, washed, and designated B cells.

Antigen-Specific Stimulation. Specific sensitization was carried out under conditions previously described (7). Briefly, 0.1-ml volumes consisting of serial dilutions of PBM were plated in flat-bottom microtiter plates (Costar, Cambridge, MA; No. 3596). RPMI 1640 medium was supplemented (9) with penicillin, streptomycin, glutamine, pyruvate, nonessential amino acids, $5 \times 10^{-5} M$ 2-mercaptoethanol, and 5% fetal calf serum (Microbiological Associates, Walkersville, MD; Lot No. 94055).

Cultures were inoculated either with 5×10^6 SRBC as the sensitizing antigen or with 10% SRBC-absorbed pooled human A serum. Antigen-free cultures were set up simultaneously. In a few experiments, hapten TNP-conjugated burro red blood cells (B-TNP) or B-TNP-absorbed human serum was used as an additional antigen. Unless indicated otherwise, all cultures received, in addition, 10% unabsorbed human serum, which was added 16–20 hr after the start of culture, and 0.003% heat-killed *Staphylococcus aureus* of Cowan strain I (American Type Culture Collection No. 12598) as a B-cell mitogen (10, 11). B-cell differentiation factor (BDF) from supernatants of lipopolysaccharide-stimulated adherent human PBM cells (12) was also added at a final concentration of 10%. Cultures were fed daily with 10 μl nutritional cocktail (9). After 7 days in culture, the cells were harvested and tested for anti-SRBC plaque-forming cells (PFC) in a modified Jerne hemolytic plaque assay (13). The PFC response in cultures not treated with antigen usually did not exceed 10% of the response of specifically sensitized cultures. The background response is not subtracted in the data reported here.

Absorption of Human Serum. Heat-inactivated serum was absorbed on ice twice with packed SRBC or BRBC-TNP at a ratio of 2:1 (v/v) for 0.5 hr and stored at −20° C until used.

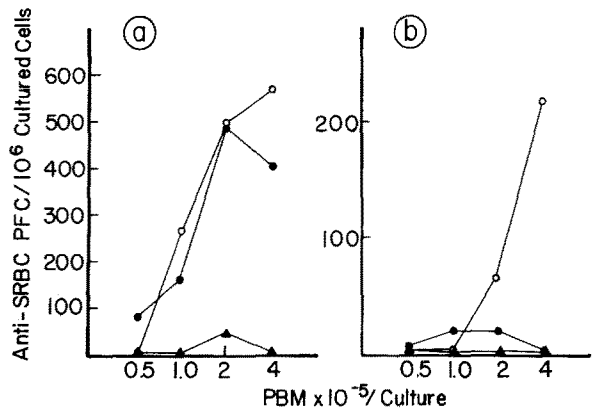

Fig. 1. Immunogenicity of SRBC-absorbed human serum. Anti-SRBC PFC responses of two donors (a and b) whose PBM were immunized with intact SRBC (•) or with 10% SRBC-absorbed serum (○) or cultured without antigen (▲).

Addition of Concanavalin A and Pokeweed Mitogen. In certain experiments effects of the mitogens Con A and PWM were tested in our culture system. These mitogens were added either at culture initiation or 2 days later. In the cultures, the final concentration of Con A was 10 μg/ml and that of PWM was 10 μl/ml; other culture conditions were changed. The effect of early and delayed addition of PWM to cultures in which *S. aureus* bacteria were omitted was also tested.

RESULTS

Antigenicity of SRBC-Absorbed Human Serum

PBM were cultured under conditions previously described (7). One set of cultures was immunized with intact SRBC, and another with SRBC-absorbed human serum. Data shown in Fig. 1 reveal the response of two PBM donors (a and b), one of whom (b) was a poor responder to particulate SRBC antigen. SRBC-absorbed serum induced anti-SRBC PFC responses in cultures from both donors; PBM from the donor that responded poorly to intact SRBC responded significantly better to antigen in SRBC-absorbed serum. Titration experiments were performed to quantify solubilized antigen in absorbed serum. Routinely, 10% unabsorbed serum was added 16–20 hr later to these cultures. Examples of results obtained with two batches of sera absorbed independently with SRBC are shown in Fig. 2. Peak anti-SRBC PFC responses were elicited with 10% (v/v) absorbed serum added to PBM cultures of two different donors. A similar pattern

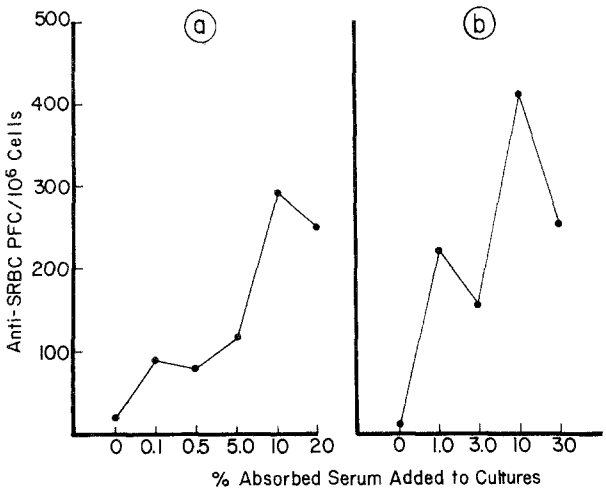

Fig. 2. Quantitation of antigen in absorbed serum. Anti-SRBC PFC responses of two PBM donors (a and b) whose cells were immunized with varying concentrations of two batches of SRBC-absorbed human serum.

of response was also obtained if absorbed serum, mixed with unabsorbed serum of the same donor, was added at culture initiation so as to keep the serum concentration in cultures constant; delayed addition of unabsorbed serum was omitted in the latter cultures.

The PFC response induced by antigen-absorbed serum was found to be T cell dependent (Table I). It was also specific for the antigen used for serum absorption as shown by the use of SRBC- and BRBC-TNP-absorbed serum. With SRBC-absorbed serum, only anti-SRBC responses were elicited. The observation that anti-TNP responses could be obtained by absorbing serum with BRBC-TNP conjugates shows that haptenic antigen is also immunogenic in solubilized form.

Addition of PWM to the Culture System

The control of suppressor T cells is, in our experience, a critical factor for eliciting antigen-specific PFC responses. Activation of antigen-responsive B cells is inhibited at high PBM cell densities because of an abundance of suppressor cells, whereas in low cell-density cultures a deficiency of helper T cells was found to be a limiting factor. The T-cell mitogen Con A can effectively be used to amplify effects of helper and suppressor T cells in this culture system (7). Early addition of Con A (at culture initiation) consistently abrogates antibody formation except in very low-density PBM cultures, where suppressor T-cells activity is suboptimal. With delayed addition of Con A (48 hr after culture initiation), PFC responses are regularly enhanced.

PWM, also shown to be a T-cell mitogen [besides being mitogenic for B cells (15)], was tested under similar conditions. It was found that PWM effects (similar to those of Con A) were dependent upon the cell density and the time of addition. PWM inhibited antigen-specific responses when added early and enhanced them when added late (Fig. 3). Suppression was most pronounced in high cell-density cultures and enhancement was best seen in low cell-density cultures. Using SRBC-absorbed serum as a source of antigen, PWM added at culture initiation was generally less inhibitory under these conditions than in cultures immunized with particulate SRBC antigen. An example of the effect of adding PWM at culture initiation using either intact SRBC or absorbed serum as antigen is illustrated in Fig. 4.

Effects of early and delayed addition of PWM could be appreciated only in cultures with *S. aureus* added at culture initiation. When *S. aureus* was omitted, PWM induced a response only when added at culture initiation; no responses were elicited when PWM was added on day 2 (Table II).

DISCUSSION

In this study, we have utilized a recently introduced system (7) for studying antigen-specific

Table I. Antigen Specificity of Absorbed Serum[a]

Responding cells	Antigen	(PFC/10^6 cultured B cells) Anti-SRBC	Anti-TNP
B + T (1:1)	SRBC + B-TNP	136	248
	SRBC-absorbed serum	604	32
	B-TNP-absorbed serum	40[b]	352
	—	92[b]	36
B	SRBC-absorbed serum	0	0
	—	0	0
T	SRBC-absorbed serum	0	18
	—	12	8

[a]Cells were cultured at concentrations of 1×10^5 and/or 1×10^5 T cells per culture (0.1 ml) for 7 days in the presence or absence of antigen as shown. B cells here designate the non T-cell fraction obtained after depletion of E rosette-forming cells and monocytes. Antigens used were either washed SRBC or BRBC conjugated with hapten TNP (B-TNP), each added to the cultures at a concentration of 0.03%, or absorbed human serum (absorbed with SRBC or B-TNP) added to the cultures at a centration 10%. For each PFC assay, cells from eight microwells were pooled and washed; an aliquot was tested for plaque formation and the observed value was multiplied by the dilution factor to give the final result.

[b]The relatively high response in the absence of SRBC is perhaps partly related to the fact that T cells were obtained by SRBC rosetting and that residual contaminating SRBC fragments were present in the T-cell suspensions that were added to the B cells.

Table II. Addition of PWM to Antigen-Stimulated PBM Cultures in the Presence and Absence of *S. aureus*[a]

Expt	*S. aureus*	PWM	Anti-SRBC PFC/10^6 cells
1	+	Day 0	125
	−		159
	+	Day 2	290
	−		0
2	+	Day 0	6
	−		80
	+	Day 2	433
	−		0
3	+	Day 0	96
	−		124
	+	Day 2	210
	−		12

[a]PBM cultures were sensitized with SRBC antigen under conditions described in the text, with the exception that *S. aureus* was omitted in some cultures. PWM, 10 μl/ml, was added to the cultures either at culture initiation (day 0) or 48 hr later (day 2).

humoral immune responses of human PBM *in vitro*. Essential ingredients of this culture system are the presence of human serum and heat-inactivated *Staphylococcus aureus* bacteria (Cowan strain) in addition to the antigen which is added in particulate form.

A widely used system in which B cells are allegedly stimulated nonspecifically to generate anti-SRBC PFC is that described by Fauci and Pratt (5, 6). Necessary ingredients of the latter system are human serum and the polyclonal B-cell activator pokeweed mitogen (PWM). An essential feature of the Fauci-Pratt system is the necessity for multiple absorptions of the human serum with SRBC. It was suggested that absorption with antigen removes antigen-specific inhibitory factors. In view of the data presented here it would appear that immunogenic material is shed from the SRBC during absorption of the serum, and we contend that solubilized antigen, rather than removal of inhibitory factors, accounts for the influence of the absorption of serum with the antigen being tested.

Our data provide the bases for an assessment of principles of the humoral immune response of human PBMs *in vitro*. Earlier, it was shown that because of the cellular composition of peripheral blood lymphocytes, effective B-cell activation requires a carefully balanced stimulation of B cells and T cells (7). Antigen-specific activation of B cells was found to be T cell dependent on the one hand, but highly sensitive to the activity of suppressor cells on the other. The response could be completely inhibited by adding concanavalin A at culture initiation (due to suppressor-cell activity). We show here that the same applies for PWM. The enhancement effect of the delayed addition of PWM (like the delayed addition of Con A) could be demonstrated only when *Staphylococcus aureus* was used to initiate the response of B cells. If PWM was used as a sole stimulator, it had to be added at the start of culture for the initiation of B-cell responses, a time at which PWM also activates the response of suppressor cells, which subsequently cut short the antigen-specific response of B cells.

The ease of inducing antigen-specific responses with absorbed serum suggests that antigen fragments (as contained in absorbed serum) rather than the intact sheep erythrocytes are immunogenic in the *in vitro* system. The addition of soluble antigens, rather than of intact red cells (whose solubilization would consume time), should allow a more immediate initiation of the PFC response, thus enabling B-cell responses to start before suppressor cells reach their full activity. Thus, using the antigen-absorbed serum in the PWM response, antigen-specific responses might be already under way at the time when PWM-induced suppressor cells become activated. This interpretation is, in fact, supported by our finding that the inhibitory activity of PWM added early to *S. aureus*-stimulated PBM cultures is generally less effective in cultures stimulated with solubilized antigen than in cultures stimulated with intact SRBC.

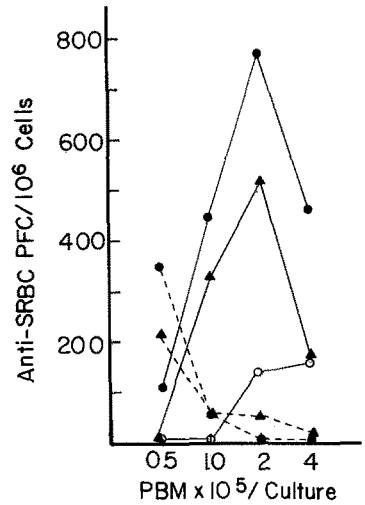

Fig. 3. Effect of Con A and PWM on antigen-specific responses. PBM cultures were immunized with SRBC and received PWM (•) or Con A (▲) either at culture initiation (dashed line) or 48 hr later (solid line). The response of PBM cultures in the absence of PWM or Con A is shown by open circles (○). Responses in antigen-free cultures were less than 10% of those in antigen-stimulated cultures (not shown).

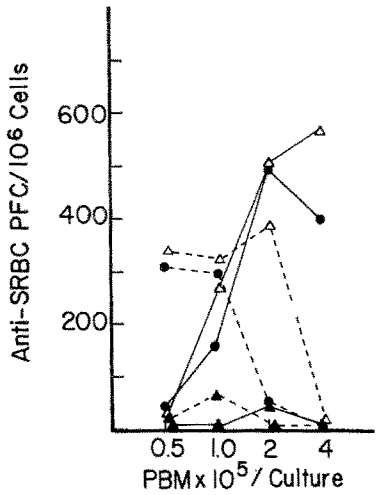

Fig. 4. Inhibitory effect of PWM on antigen-specific responses. PBM cultures were immunized with intact SRBC (•) or 10% SRBC-absorbed serum (△) and cultured in the absence (solid line) or presence (dashed line) of PWM added at culture initiation to either antigen-stimulated or antigen-free (▲) cultures.

S. aureus as utilized for these experiments has been reported to be an exclusive B-cell mitogen (14). With PWM, in contrast, T cells as well as B cells are strongly activated (15), and we contend that it is the activation of suppressor T cells that most likely results in abortive responses to antigen. This explanation would perhaps account for the apparent "nonresponders" which have been shown to manifest excessive suppressor cell activity in the Fauci-Pratt system (16).

Antigen-specific humoral immune reactions of human PBM *in vitro* have long been considered difficult to achieve. In this context, we have previously shown that a major obstacle has been the control of suppressor cells. Our data presented here suggest that it may also be the dominating effect of suppressor cells that might make B-cell responses in the Fauci-Pratt system appear different from B-cell responses in our system. We contend that both systems measure antigen-specific responses. The main difference may be that of the two mitogens used to initiate B-cell responses (PWM and *S. aureus*), PWM may be the stronger activator of regulatory T cells.

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