ACTIVATION OF HUMAN B LYMPHOCYTES

I. Direct Plaque-Forming Cell Assay for the Measurement of Polyclonal Activation and Antigenic Stimulation of Human B Lymphocytes

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Understanding the events involved in the activation of human bone marrow-derived (B) lymphocytes to antibody formation and secretion has been hampered by the lack of a simple reproducible method of culture and assay. In the mouse model, induction of primary in vitro antigenic stimulation (1), as well as polyclonal B-cell activation (2, 3), with measurement of single cell antibody production by plaque-forming cell (PFC)[1] assays has allowed for in depth probing of the mechanisms of B-cell activation. Such a simple and reproducible methodology has not been available for human studies. A few reports have appeared describing primary in vitro activation of human B lymphocytes from various organs, particularly tonsil, by soluble or particulate antigens with subsequent measurement of antibody response by a PFC assay (4–8). However, these studies have been quite difficult to reproduce, and subsequent follow-up studies have not appeared. In addition, recent studies have pointed out a potential area of significant artifact in primary in vitro stimulation of human cultures with sheep red blood cells (SRBC) (9). Attempts to establish PFC assays after polyclonal activation of human B lymphocytes have met with similar difficulties (10). No reproducible studies have appeared which demonstrate single cell antibody production by human lymphocytes after activation with polyclonal B-cell activators as measured by a PFC assay.

The present report describes the conditions necessary for the in vitro induction of polyclonal activation as well as antigenic stimulation of human tonsillar lymphocytes with measurement of antibody production by a direct PFC assay. The methods are simple and the results reproducible. Critical factors for induction and assay are delineated and areas of potential artifact are described. This system can be readily employed to explore the complex events associated with human B-lymphocyte activation.

Materials and Methods

Tonsils. Tonsils were obtained from subjects undergoing routine tonsillectomy for chronic tonsillitis. Tissue was placed immediately in ice-cold RPMI-1640 (Grand Island Biological Co.,

[1] *Abbreviations used in this paper:* FCS, fetal calf serum; FITC, fluoresceinated isothiocyanate; HBSS, Hanks' balanced salt solution; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; PFC, plaque-forming cell; PPD, purified protein derivative of tuberculin; PWM, pokeweed mitogen; SIII, pneumococcal polysaccharide; TNP, trinitrophenyl.

Grand Island, N. Y.) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin sulfate. Processing of cell suspensions was always begun within 30 min of surgical removal of the tonsils.

Cell Suspensions. Tonsils were teased apart with forceps in a Petri dish in cold RPMI-1640 and clumps dispersed by repeated aspirations through a pipette. Debris was removed by passing the cell suspension through sterile gauze followed by further purification using standard Ficoll-Hypaque density centrifugation (11). Differential counts of the cell suspensions were done on cytocentrifuge preparations stained with Wright's stain.

Culture Conditions. Cultures for the determination of antibody synthesis were performed in a modified Mishell-Dutton system (1). The precise requirements for optimal culture conditions in this system, as well as the effect of variation of factors such as media, sera, cell density, and time in culture will be described below. Basically, cultures were performed as follows: cultures were set up in multi-well (1.5-cm diameter wells), flat bottom plastic plates (Linbro Scientific Inc., New Haven, Conn.). 5×10^6 cells in 2 ml of RPMI-1640 containing 1% trypticase soy broth, 0.02 M L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate were placed into each well. Cell densities ranging from 0.1 to 10×10^6 were tested and 5×10^6 proved optimal. Cultures were evaluated with serum-free media as well as with serum supplements at various concentrations. Two main categories of sera were tested: fetal calf serum (FCS) obtained from International Biological Laboratories Inc., Rockville, Md. and from Colorado Serum Co., Denver, Colo.; and various lots of pooled human AB serum obtained from International Biological Laboratories Inc. and University Plasma Center, College Park, Md., as well as sera from individual AB donors. All sera were heat inactivated at 56°C for 45 min, passed through Millipore filters, and either used directly or after repeated absorptions with SRBC.

In every experiment, cultures were stimulated with either pokeweed mitogen (PWM) lot 153601 or 254004 (GIBCO) in a wide concentration range, or SRBC in various concentrations ranging from 0.1 to 10×10^6 per culture. In some experiments cultures were stimulated with one of several substances known to be B-cell activators in mouse culture systems (3). These included *Escherichia coli* 0 128:B12 lipopolysaccharide (LPS), lot 617124 (Difco Laboratories, Detroit, Mich.); purified protein derivative of tuberculin (PPD), lot A10 (Connaught Research Laboratories, Toronto, Canada); keyhole limpet hemocyanin (KLH), lot SPH 95 (Pacific Bio-Marine Supply Co., Venice, Calif.); dextran sulfate ($DxSO_4$) (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden); and pneumococcal polysaccharide (SIII) (gift of Dr. Phillip J. Baker, National Institutes of Health, Bethesda, Md.).

Cultures were incubated for durations of 1–10 days on a rocker platform (7 cycles/min) at 37°C in 5% CO_2 in air at 100% humidity. At the end of the culture period, cells were harvested from the plates by gentle scraping with a rubber policeman followed by repeated aspirations through a Pasteur pipette. Cells were transferred to plastic tubes, washed twice in cold RPMI-1640, and brought to the appropriate concentration for the plaque assay. Cultures for the determination of blast transformation as measured by tritiated thymidine incorporation were set up in microtiter plates and performed as previously described (12).

Assay for PFC. Cell suspensions from cultures stimulated either with polyclonal B-cell activators such as PWM or particulate antigen such as SRBC were assayed for direct PFC against SRBC by a modification of the Jerne-Nordin hemolysis-in-gel method (13) employing an ultra-thin layer gel technique. After heating to boiling, 0.7 ml of 0.5% Noble agar (Difco Laboratories) in Hanks' balanced salt solution (HBSS) (NIH media supply section) containing 0.05% DEAE-dextran (Pharmacia Fine Chemicals Ltd.) was added to Falcon tubes (no. 2052; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and kept at 46°C. Thereupon, 0.05 ml of indicator red cells diluted 1:5 in HBSS (20% suspension), 0.2 ml of the cell suspension in the appropriate concentration, and 0.05 ml of guinea pig serum (previously absorbed with SRBC) diluted 1:4 in veronal-buffered saline were added to each tube (14). The mixture was then plated as follows: three separate 0.2 ml drops were placed on plastic Petri dishes (no. 1001; Falcon Plastics, Div. of BioQuest). A 22 × 32 mm glass cover slip (Exax No. 1 cover glass; Kimble Glass Company, Toledo, Ohio) was immediately placed on each drop. By capillary action, the drop spread into an ultra-thin layer beneath the cover slip. Plates were incubated for 3 h at 37°C and PFC were counted on a dissecting microscope using indirect light.

In some experiments the SRBC were haptenated with one of several haptens in an attempt to increase the sensitivity of the assay for polyclonal B-cell activation after PWM stimulation. The 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) hapten and the fluoresceinated isothiocyanate

(FITC) hapten were conjugated to SRBC as previously described (15), and trinitrophenyl (TNP) haptenation was carried out as previously reported (16).

Inhibition of PFC. The functional and metabolic requirements for antibody synthesis and secretion were determined by subjecting the cultures to a variety of inhibitory manipulations (17). In some experiments cells were heat killed at 56°C for 30 min before assay. In others, complement (C) was not added, or the plaquing assay was conducted at room temperature or at 4°C instead of at 37°C. In a group of other experiments, 24 h before harvesting, cell cultures were either irradiated with 5,000 R or one of the following inhibitors were added: mitomycin C, 4 and 40 μg/ml (Sigma Chemical Co., St. Louis, Mo.); puromycin, 1 and 10 μg/ml (Sigma Chemical Co.); actinomycin D, 0.5 and 5 μg/ml (Sigma Chemical Co.); and cycloheximide, 1 and 10 μg/ml (Sigma Chemical Co.).

Addition of Allogeneic Cells. In some experiments, 1×10^6 mitomycin C-treated, Ficoll-Hypaque-separated peripheral blood mononuclear cells from an unrelated donor were added to the cultures at day 0. This was done in order to determine the effect of a simultaneous mixed leukocyte reaction (in vitro allogeneic effect) on ongoing antibody synthesis.

Results

Cell Suspensions. After Ficoll-Hypaque gradient centrifugation of the cell suspensions from both tonsils of a single individual, the mean ± SEM yield of mononuclear cells from 35 subjects was $1.5 \pm 0.3 \times 10^9$. Cell viability as measured by trypan blue dye exclusion was always greater than 90%. The cell suspensions contained 91 ± 0.7% lymphocytes, 5 ± 0.6% lymphoblast-like cells, 3.8 ± 0.4% monocytes, and from 0 to 2% plasma cells.

Culture Conditions. A cell density of 5×10^6 cells per well was optimal and peak PFC responses were usually found with PWM concentrations between 1:20 and 1:1,000 final dilutions. In cultures stimulated with SRBC, concentrations of $1\text{–}5 \times 10^6$ SRBC per well were optimal. Addition of HEPES buffer or feeding of cultures with nutrient cocktail was not necessary. In none of the experiments was a substantial PFC response detectable during the first 2 days of culture. By 3 days small responses were detected which peaked sharply at 5–7 days and began declining by 8 days.

The nature of the serum supplementation was clearly of utmost importance. All lots of pooled human AB sera were freshly drawn as were sera from individual AB donors. Cells did not survive and no PFC were detected if serum was not added to cultures. The addition of 2-mercaptoethanol did not substitute for serum. Background PFC were higher with FCS than with most lots of human AB serum and concentrations of 10% human AB serum proved optimal. An observation made during the course of these studies was that absorption of serum with SRBC before culture removed a suppressant factor from the serum and increased (up to 20-fold) the ability of that serum to support the PFC response of cultures activated with various stimuli. This enhancement of PFC response was seen without significantly increasing background PFC. This phenomenon will be described below.

Assay for PFC. The combined gel-cover slip method proved to be sensitive, reproducible, and quite simple to perform and read. The clarity of the plaques, as well as the demonstration of a single mononuclear cell in the center of a plaque is shown in Fig. 1. It is important to emphasize that in the first several experiments a mononuclear cell was identified in the center of every plaque before calling it a true plaque. Subsequently, in all experiments representative plaques on every plate were checked for a cell in the center. Such an approach was felt to be essential in order to rule out artifact since, in previous studies,

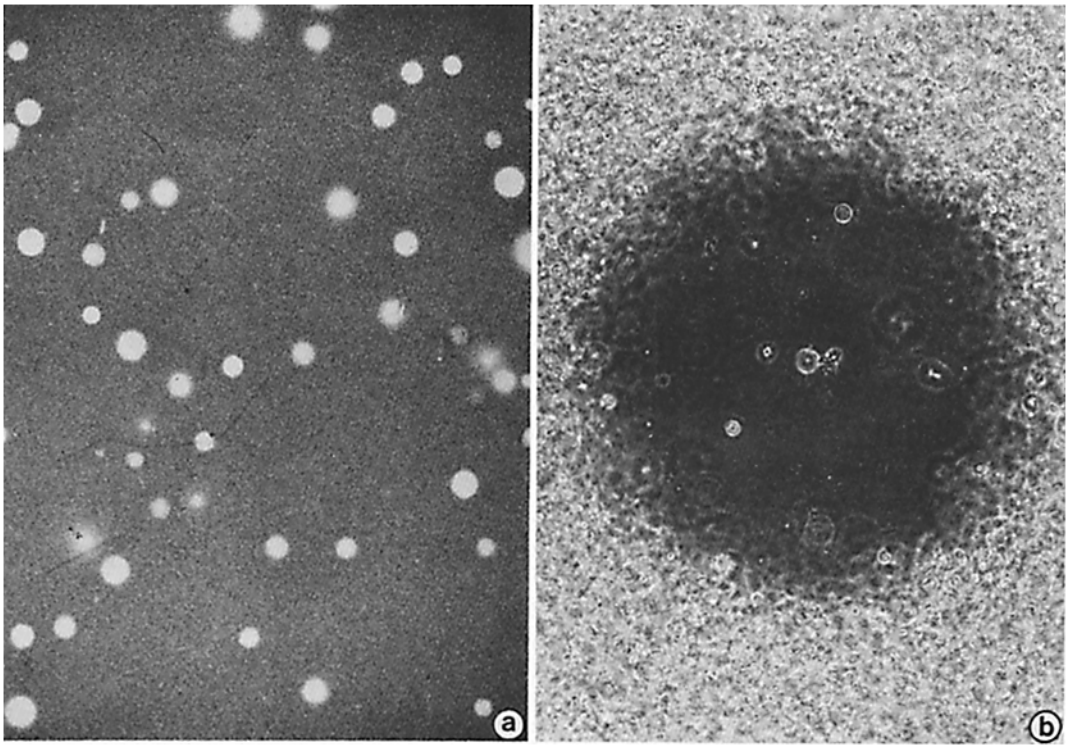

FIG. 1. (*a*) Appearance of plaques in ultra-thin agar gel layer beneath a glass cover slip. × 6.5. (*b*) Single mononuclear cell in the center of a typical plaque from a PWM-stimulated culture of human tonsillar lymphocytes. × 200.

there were considerable difficulties in obtaining plaques in cultures of human lymphocytes. In addition, under certain conditions in which SRBC were used as the stimulus, artifactual plaques could be demonstrated as will be discussed below. Plaques were detectable by 30 min and reached maximal clarity and number by 2–3 h.

Demonstration of Artifactual Plaques. As has recently been pointed out by Muchmore et al. (9), human lymphocyte cultures stimulated with SRBC in which the serum or plasma supplement has not been absorbed with the immunizing SRBC can result in artifactual plaques. We have found this to be the case in our culture system. The mechanism of formation of artifactual plaques is an agglutination of stimulator SRBC in culture by anti-SRBC antibody present in the serum supplement. When the lymphocytes are prepared for the plaque assay, these aggregates are carried over and after setting in the gel, the antibody diffuses out symmetrically, attaching to the indicator SRBC. Upon addition of C, a plaque is formed which usually does not have a mononuclear cell in the center. Thus, absorption of sera with SRBC was essential from two standpoints, the removal of blocking factors mentioned above, and the elimination of artifactual plaques (Table I).

Inhibition of PFC. Formation of true plaques was inhibited by greater than 95% by heat killing of cells at 56°C before assay. Withholding of C from the assay

TABLE I
Effect of Absorption of Human AB Serum with SRBC on the PFC Response to PWM and SRBC Stimulation

Stimulus	True plaques		Artifactual plaques	
	Unabsorbed* serum	Absorbed serum	Unabsorbed serum	Absorbed serum
None (background)	1‡	9	0	0
PWM	15	210	5	0
SRBC	9	148	2,850	0

* 10% human AB serum used in all cultures.
‡ PFC/10^6 lymphocytes.

resulted in complete abrogation of plaques. Plaques in assay plates incubated at room temperature rather than 37°C were decreased by approximately 50% and were smaller and less clear. Incubation at 4°C resulted in greater than 95% suppression of PFC. Irradiation of cultures with 5,000 R the day before assay resulted in greater than 75% suppression of PFC numbers. The addition of inhibitors which affect protein synthesis such as puromycin, cycloheximide, and actinomycin D 24 h before assay in concentrations which did not significantly reduce viability resulted in from 50 to 90% suppression of PFC numbers. Mitomycin C at a concentration of 4–40 μg/ml, when added at day 0 resulted in death of the cultures by day 5. When added 18 h before harvesting, it did not affect the number of PFC.

PFC Response to PWM Stimulation. The anti-SRBC response elicited by the polyclonal activation of tonsillar lymphocytes by PWM as compared to the response to specific stimulation by SRBC is shown in Fig. 2. It is of interest that when the conditions described above for avoiding artifactual plaques after stimulation with SRBC were met, the magnitude of the anti-SRBC response after polyclonal activation by PWM was as great or greater than that after antigenic stimulation with SRBC.

In several experiments, an attempt was made to increase the sensitivity of the PFC assay after PWM stimulation by coupling various haptens such as TNP, NIP, and FITC to the target SRBC. There was no difference in numbers of PFC when TNP-SRBC was used as the indicator as opposed to unconjugated SRBC. The coupling of NIP or FITC haptens to SRBC resulted in approximately a 30–40% increase in numbers of detectable PFC in a few assays, but this was not consistent.

Allogeneic Effect. The effect of adding 1×10^6 mitomycin C-treated Ficoll-Hypaque-separated peripheral blood mononuclear cells from an unrelated donor to the 5×10^6 tonsillar lymphocytes per well on the PFC response to SRBC or PWM stimulation is shown in Fig. 3. The number of background PFC was unchanged ($P > 0.2$, Student's t test). There was a variable and nonsignificant ($P > 0.2$) effect on the PWM-stimulated cultures, although there was clearly suppression seen in some experiments. In contrast, there was a significant enhancing effect ($P < 0.05$) on the SRBC-stimulated cultures.

Blast Transformation and PFC Response. In PWM-stimulated cultures, peak blast transformation consistently preceded peak PFC response by 1 to 2

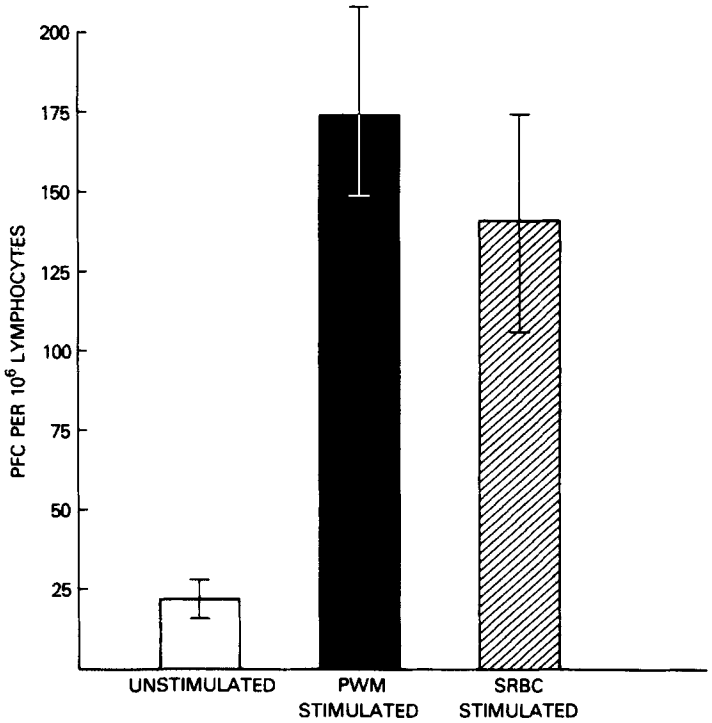

FIG. 2. PFC response of human tonsillar lymphocytes after stimulation with either PWM or SRBC. Data represent the mean ± SEM response in 23 tonsils.

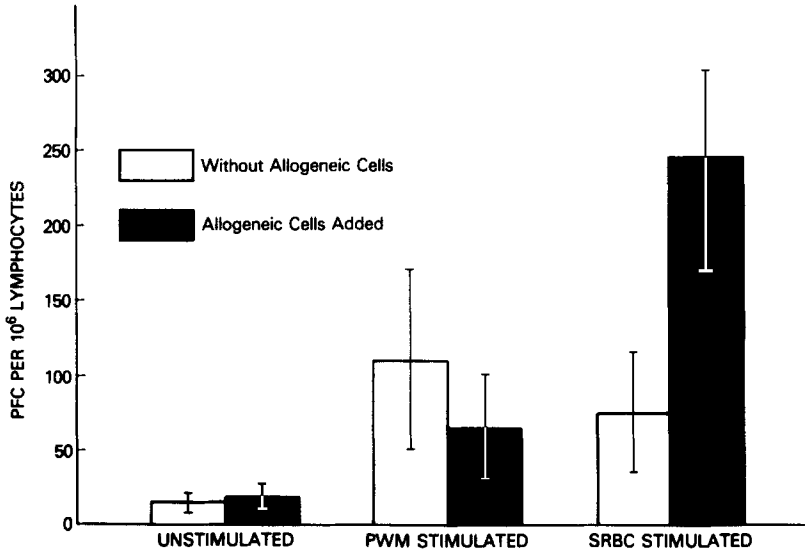

FIG. 3. Allogeneic effect on PFC response of human tonsillar lymphocytes. 1×10^6 mitomycin C-treated peripheral blood mononuclear cells from an unrelated donor were added to 5×10^6 tonsillar lymphocytes on day 0 and cultures were stimulated with either PWM or SRBC. Assay done on day 5. Data represent the mean ± SEM response in eight tonsils.

TABLE II
PFC Response of Human Tonsillar Lymphocytes to Various B-Cell Activators

Stimulus	PFC/10^6 lymphocytes
Background	13 ± 5*
LPS	82 ± 21
PPD	91 ± 33
KLH	21 ± 5
SIII	9 ± 3
$DxSO_4$	3 ± 2

* Mean ± SEM of 10 tonsils.

days. Stimulation with SRBC rarely resulted in a detectable blast transformation response of greater than three to four times above background.

PFC Response to Other Polyclonal B-Cell Activators. The PFC responses of human tonsillar lymphocytes to various substances known to be B-cell activators in mice are shown in Table II. Stimulation with LPS and PPD gave significant increases over background ($P < 0.01$ and $P < 0.05$, respectively). Optimal stimulatory concentrations varied among different tonsils. In general, peak PFC responses were seen at concentrations of LPS from 50 to 500 μg/ml and PPD from 10 to 50 μg/ml. When mean responses of 10 subjects were compared, KLH stimulation did not result in a significant PFC response ($P > 0.2$). However, certain individuals manifested a substantial increase in PFC response over background after stimulation with KLH. SIII and $DxSO_4$ did not activate cultures to PFC responses. Of interest was the fact that none of these substances stimulated cultures significantly to blast transformation at 3 or 5 days as measured by tritiated thymidine incorporation except for KLH, which occasionally resulted in a blast transformation response of up to fivefold over background.

All these substances were tested for the presence of endotoxin by the limulus assay as previously described (18) (assays kindly done by Dr. Ronald J. Elin, NIH, Bethesda, Md.). LPS contained endotoxin as expected. SIII (50 μg/ml) and PPD (500 μg/ml) had nanogram quantities, while the highest used concentrations of KLH and $DxSO_4$ had no detectable endotoxin. Of note also is the fact that PWM in the concentrations used in the present studies did not contain detectable endotoxin.

Discussion

The present study clearly demonstrates that human B lymphocytes can be successfully and reproducibly activated in vitro and subsequently assayed for antibody production by a direct PFC assay against SRBC targets. This can be accomplished by specific stimulation with an antigen such as SRBC, and even more so by polyclonal activation of lymphocytes with PWM. Also, successful induction and PFC assay of antibody production follows stimulation with other substances which have been shown to be B-cell activators in the mouse (3) and which have not been consistently shown to activate B cells in humans as measured by the standard thymidine incorporation assays for blast transformation. Despite the fact that such an apparently simple system of culture and

assay would be of great interest and importance in the investigation of the mechanisms of B-cell activation in humans, surprisingly few studies using antigenic stimulation have appeared. Furthermore, the use of mitogens as a probe in the study of B-cell activation in the mouse has contributed to our understanding in this area (3). The present study makes available such a model of mitogen-induced B-cell activation in humans adaptable to the PFC assay.

The success of this model resulted from a combination of a methodical modification of the original Mishell-Dutton system (1) and almost chance discovery of certain critical factors. With regard to the culture conditions, it became evident that there was an optimal cell density, media composition, duration of culture, and concentration of stimulants. However, the most critical factor was clearly the sera employed. Some batches of serum were strongly supportive of PFC responses to SRBC stimulation as well as to polyclonal B-cell activation, and other batches resulted in very poor responses. In addition, it was found that absorption of sera with SRBC resulted in most cases in a marked (up to 20-fold) increase in the ability of that sera to support PFC responses to SRBC. Clearly, most human sera contain a factor which markedly suppresses the PFC response to SRBC targets. This factor does not influence cell yield or viability of cultures and can be removed by absorption with SRBC. Although it is probable that this factor is antibody which reacts with SRBC determinants, its precise nature is not entirely clear at present and is the subject of current investigation (B. F. Haynes and A. S. Fauci, unpublished observations). Suffice it to say that this serum inhibitory factor probably was one of the critical reasons for previous difficulties in establishing a reproducible PFC assay for in vitro antibody synthesis against SRBC in human cultures. The importance of multiple absorptions of supplemental sera with SRBC becomes even more critical in SRBC-stimulated cultures in which the use of unabsorbed serum results in a decrease in true plaques and the appearance of large numbers of artifactual plaques as described in the present study and previously by Muchmore et al. (9).

The particular hemolysis-in-gel assay used in this study lends simplicity and clarity to the system. It combines the sensitivity of a monolayer technique with the stability and clarity of gel and appears to be well suited to the human PFC assay.

The use of SRBC as stimulator and target in the PFC assay is the standard approach for primary in vitro sensitization of lymphocyte cultures (1, 17). The use of SRBC as targets for the measurement of antibody synthesis after polyclonal B-cell activation is also well established in the mouse system (3). It is based on the premise that polyclonal B-cell activators stimulate lymphocytes to secrete antibodies with wide ranges of specificities including those that react with determinants on the SRBC. It is clear from the present study that this is also applicable to the stimulation of human B cells by polyclonal activators, particularly PWM. It is noteworthy that in the human system the sensitivity of the assay is not enhanced by hapten conjugation of the SRBC to the great extent that is seen in the mouse system (3, 19). The reason for this is unclear at present, but it may merely represent fundamental species differences in the hapten specificities of the B-cell repertoire. Nonetheless, the magnitude and consistency of the PWM-induced PFC response against even unconjugated SRBC is sufficient to allow further manipulations of the culture conditions in order to

define more precisely the specific requirements for B-cell activation. It is clear, for example, that the PFC response of B cells to PWM stimulation in this system is T-cell dependent since removal of T cells from the suspensions results in abolishment of the PFC response.[2] This is consistent with the findings of Janossy and Greaves (20) in human tonsil and Keightley et al. (21) in human peripheral blood using the presence of intracytoplasmic Ig in plasma cells as their assay. In addition, the response to SRBC stimulation is enhanced by an in vitro allogeneic effect (Fig. 3) similar to that described in mice (22), further supporting the parallelism between the present model and numerous well-defined mouse studies.

The finding of polyclonal activation of B lymphocytes by substances such as LPS, PPD, and KLH is of particular interest. Such substances are known B-cell activators in mouse cultures (3), but they have not been shown to be mitogenic for human lymphocytes as measured by blast transformation. In particular, LPS has been shown to be ineffective in stimulating lymphocytes from human tonsil (23) or blood (24) to blast transformation. In the present study, blast transformation was either totally or almost totally absent after stimulation with LPS, yet substantial PFC responses were detected. Stimulation with $DxSO_4$ on the other hand resulted in neither a proliferative nor an antibody response. These findings are quite consistent with the theories that B lymphocytes are made up of distinct subpopulations at various stages of differentiation, which can respond in a variety of ways (proliferation, antibody secretion, neither, or both) to activation with different B-cell activators (3, 20, 25).

Endotoxin contamination of these B-cell activators could not entirely explain the responses seen in these studies since, when present, endotoxin was found in nanogram quantities while true endotoxin stimulation with LPS required concentrations of 50 μg/ml or greater for detectable PFC responses. In addition, SIII contained endotoxin and did not give significant PFC responses while KLH contained no detectable endotoxin, yet resulted in substantial PFC responses in some individuals. Finally, substances such as PPD almost always exhibited a dose-response phenomenon with suppression of PFC at supraoptimal concentrations. If endotoxin was responsible for all the PFC responses seen, one would expect a linear increase in PFC response with increasing concentrations, given the extremely small amount of endotoxin present in the contaminated substances.

Thus, these studies provide a simple, sensitive, and reproducible system of induction and assay of antibody production by human lymphocytes after antigenic stimulation, as well as polyclonal activation of B cells. This model can be readily employed to explore the complex factors, cell requirements, and interactions involved in the activation of human B lymphocytes.

Summary

A model for the detection of single cell antibody production by human tonsillar lymphocytes after stimulation with either sheep red blood cells (SRBC) or polyclonal B-cell activators has been described. The culture system is a modified Mishell-Dutton technique with certain critical factors identified. The assay is a

[2] Fauci, A. S., and K. R. Pratt. Manuscript in preparation.

sensitive and reproducible hemolysis-in-gel system employing an ultra-thin layer gel technique measuring plaque-forming cells (PFC) against SRBC targets. Several factors essential for optimal responses are described, but the critical feature of the culture system is the use of selected lots of human AB serum supplements which are extensively absorbed with SRBC. This removes a blocking factor present in most human serum which suppresses the B-cell response to SRBC targets after stimulation with either SRBC or several polyclonal B-cell activators. In addition, absorption of serum with SRBC eliminates the presence of artifactual plaques. Background PFC are extremely low and stimulated cultures show significant and reproducible responses. These studies provide a simple, sensitive, and reproducible model for probing the complex events associated with activation of human B lymphocytes.

We wish to thank Ms. Gail Whalen for expert technical assistance. We are grateful to the Department of Otolaryngology of the National Naval Medical Center, Bethesda, Md. for supplying the tonsils.

Received for publication 10 May 1976.

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