IN VITRO GENERATION OF ANTIGEN-SPECIFIC HEMOLYTIC PLAQUE-FORMING CELLS FROM HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

By JOHN MISITI and THOMAS A. WALDMANN

From the Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Over the past few years there have been many advances in our understanding of the regulatory mechanisms that govern the humoral immune response. Many of these new insights have followed the development of techniques that permit the analysis of in vitro antibody production by antigen- or mitogen-stimulated immunocompetent cells. In the mouse, detailed analysis of the mechanisms governing B cell activation has been made possible by the availability of assays measuring single cell antibody production after antigenic stimulation. In 1963, Jerne and Nordin (1) described a simple technique for elucidating individual antibody-forming cells by suspending lymph node or spleen cells from an immunized animal in a sheep erythrocyte (SRBC)[1]-agar matrix and observing the formation of distinct zones of complement-dependent erythrocyte lysis. Further sensitivity could be obtained if lymphoid cells and target erythrocytes were suspended in monolayer without a supporting matrix (2, 3). In 1966, Mishell and Dutton (4, 5) described culture conditions under which dissociated spleen cells from nonimmunized mice could be stimulated in vitro to generate an antibody response comparable with that which occurs with in vivo immunization. Although the application of these techniques has vastly enhanced our knowledge of the regulatory mechanisms controlling antibody production by murine spleen cells, application of similar methodologies to the study of human lymphoid elements has been limited. A few investigators (6–8) have reported successful activation of B cells from human tonsil. In 1973, Hoffmann et al. (6) reported the production of anti-SRBC antibody by human tonsil cells immunized with SRBC in vitro under Mishell and Dutton culture conditions and supplemented with additional peripheral blood mononuclear cells (PBMC) or endotoxin from *Escherichia coli*. In 1974, Watanabe et al. (7) demonstrated that human tonsil cells, in the presence of 2-mercaptoethanol, could be stimulated in vitro to respond to hapten-protein conjugates, and in 1976, Fauci and Pratt (8) described a model for the detection of human tonsillar plaque-forming cells (PFC) after stimulation with either SRBC or polyclonal B cell activators and assayed against SRBC using an ultrathin-layer gel technique.

However, because the only readily accessible human lymphoid tissue is peripheral

[1] *Abbreviations used in this paper:* AET, 2-amino-ethyliso-thiouronium bromide; FCS, fetal calf serum; HRBC, horse erythrocytes; PBMC, peripheral blood mononuclear cells; PFC, plaque-forming cells; PWM, pokeweed mitogen; SRBC, sheep erythrocytes.

blood, the study of human antigen-specific B lymphocyte activation is dependent on the development of an assay that will support the in vitro immunization of peripheral blood lymphoid elements. Although established assays for measuring the polyclonal production of antigen-nonspecific antibody by human PBMC are available, there have been only few reported successes with antigen-specific systems. Fauci and Pratt (9) described a culture and assay system for the stimulation of human PBMC and subsequent measurement of direct PFC against SRBC by a hemolysis in-gel technique. However, this system required the addition of the polyclonal activator pokeweed mitogen (PWM). Luzzati et al. (10) also reported that a specific antibody response directed against SRBC could be induced in vitro. However, this system also required the addition of a nonspecific activating agent, Epstein-Barr virus. Hoffmann (11) has generated a PBMC antigen-specific response that requires both a proliferative stimulus such as heat-inactivated *Staphyloccocus aureus* and the addition of monocyte-conditioned medium containing a macrophage-produced B cell-differentiating factor, and Delfraissy et al. (12) described a method for the induction of a primary in vitro antigen-specific antibody response by PBMC using the hapten trinitrophenyl conjugated to a carrier, polyacrylamide beads, which possess polyclonal stimulatory properties. Alternatively, Dosch and Gelfand (13) have succeeded in generating an antigen-specific primary humoral immune response against both ovalbumin and SRBC without the addition of other stimulatory agents. However, their assay entails PFC enumeration on poly-L-lysine-coupled erythrocyte monolayers in Microtest II plates. Such an assay is technically difficult and supports the generation of exceptionally small plaques.

It is the purpose of this paper to describe a method for the creation of macroscopic hemolytic plaques by antigen-specific PFC generated from human PBMC that were sensitized in vitro with SRBC in the absence of nonspecific stimulatory agents. The induction of PFC was dependent on the presence of adherent cells and a radiosensitive helper T cell as well as appropriate B cell precursors in the culture system.

Materials and Methods

Materials. Wash media consisted of a balanced salt solution lacking Mg^{++}, Ca^{++}, and plasma or other protein source. Culture media consisted of RPMI 1640 with 25 mM Hepes buffer supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), and 10% heat-inactivated human plasma. Heparinized plasma for use in the culture media was obtained from donors with untreated common variable hypogammaglobulinemia and was heat inactivated at 56°C for 30 min. Plasma from four different patients was tested, and all four were capable of supporting PFC generation. Hypogammaglobulinemic plasma was used to avoid false plaques or the requirement for preabsorption with SRBC. However, when plasma from normal donors was tested, comparable results were obtained. In contrast, fetal calf serum (FCS) could not be substituted for human plasma at any stage in the cell culture or separation procedure because it led to suppression of the PFC response. In most cases, plasma was used unabsorbed. However, where the effect of absorption with SRBC was to be analyzed, plasma was first heat inactivated and then serially absorbed three times at 4°C with washed packed SRBC at a ratio of 1:9, packed SRBC:plasma, respectively. After absorption residual erythrocytes were separated from the plasma by centrifugation at 2,250 *g* for 10 min. SRBC and horse erythrocytes, the antigens used in the present study, were washed three times in phosphate-buffered saline (pH 7.4) before addition to the culture. Various numbers of erythrocytes were added to the cultures in a final volume of 100 μl. Lyophilized guinea pig complement (Flow Laboratories, Inc., Rockville, Md.) used for the

development of plaques was rehydrated, absorbed with SRBC at a ratio of 7:1 (vol:vol), complement to packed erythrocytes, stored at −20°C, and used undiluted.

Cell Preparation and Induction of PFC. To obtain the cells for in vitro generation of antibody, heparinized human peripheral blood diluted with an equal volume of a balanced salt solution lacking Mg^{++} and Ca^{++} was layered over 4.5 ml of Ficoll-diatrizoate solution (Bionetics Laboratory Products, Litton Bionetics, Inc., Kensington, Md.) in 15-ml plastic centrifuge tubes (model 2095; Falcon Labware, Oxnard, Calif.), and centrifuged at room temperature for 30 min at 550 *g*. PBMC were collected from the interfaces, washed three times with wash media, and resuspended in culture media at the appropriate cell concentration discussed below. These human PBMC suspended in culture media were cultured in 1-ml vol in sterile 35-mm tissue culture dishes (model 25000; Corning Glassworks, Science Products Div., Corning, N. Y.) with or without antigen. We found that the size and shape of the culture vessel were critical. No response was observed when PBMC were cultured in microtiter plates or round-bottomed tubes.

Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Throughout the incubation period, cultures were rocked on a Bellco rocker platform (model 7740; Bellco Glass, Inc., Vineland, N. J.) with an oscillation rate of 7 cycle/min, and supplemented daily with 75 μl of culture media containing 10% human plasma from a patient with common variable hypogammaglobulinemia. After incubation for periods indicated in the Results, cultures were transferred to 15-ml plastic tubes (model 2057; Falcon Labware) and centrifuged at 550 *g* for 15 min. The cell pellets were washed twice and resuspended in 200 μl RPMI 1640 supplemented with 25 mM Hepes buffer. At the time of plaquing, these cells were mixed with 20 μl of complement, 20 μl of heat-inactivated FCS (Reheis, Phoenix, Arizona), various concentrations of packed erythrocytes, and various volumes of 1.12% agarose (Sea Plaque, Marine Colloids, Inc., Rockland, Maine), preheated to 43°C. The optimal concentration of packed erythrocytes and agarose needed for the preparation of a uniform erythrocyte monolayer varied with erythrocytes from different species. Appropriate concentrations of erythrocytes and agarose had to be defined for each type of target cell employed. We found that 40 μl of SRBC adjusted to a hematocrit of 30 with 30 μl of 1.12% agarose, and 40 μl of horse erythrocytes (HRBC) adjusted to a hematocrit of 30 with 5 μl of 1.12% agarose added to the cultured cell suspensions described above resulted in the formation of homogeneous SRBC and HRBC monolayers, respectively.

Enumeration of PFC. PFC were enumerated using a modification of the Cunningham technique (2, 3). Shallow chambers were constructed by applying a piece of ¼-in-wide double-coated tape (Scotch brand 666; Minnesota Mining & Mfg. Co., St. Paul, Minn.) vertically down the center and at each end of a clean 75- × 25-mm unfrosted microscope slide (model 75001; Kimble Products, Toledo, Ohio), dividing the slide into two rectangles of equal area. A second slide was pressed firmly onto the first slide covering both chambers. Using a 1-cm^3 syringe and a 26-gauge needle, each chamber was filled with ~100 μl of the cell suspension. After gelation, untaped edges of the chambers were sealed with molten paraffin-petrolatum (Fisher Scientific Co., Pittsburgh, Pa.), premixed at a ratio of 3:1, paraffin to petrolatum, to prevent drying. The chambers were incubated at 37°C for 2–3 h allowing for the development of macroscopic plaques. The number of hemolytic plaques generated on the coded slides was enumerated by microscopic inspection at magnification × 40. Only those plaques in which a central cell could be identified were counted.

A reverse hemolytic plaque technique was used to measure the total number of IgM-secreting cells in the SRBC stimulated cultures. Such reverse plaques were assayed on a monolayer of protein A-treated SRBC (protein A/*Staphylococcus aureus*; Pharmacia, Uppsala, Sweden). The protein A was coupled to SRBC with $CrCl_3$ as previously described (14). The assay procedure used was identical with that described above for antigen-specific plaques with the exception that 20 μl of undiluted rabbit anti-human immunoglobulin with specificity for IgM was also added to the assay system in order to quantiate all cells producing IgM immunoglobulin molecules, and in so doing, to determine whether the SRBC were acting as a polyclonal activator.

In studies addressing the issue of whether the plaques reflected false plaques or de novo protein synthesis of specific antibody, cycloheximide at a final concentration of 10 μg/ml was

added 12 h before harvesting the cells and at the time of plaquing. Cell viability assessed by trypan blue exclusion was not effected when cultures were treated in this fashion.

Adherent Cell Depletion and Enrichment. Adherent cell-depleted and -enriched populations were prepared in order to define the effects of varying concentrations of such cells on the in vitro generation of PFC. These populations were prepared according to the method of Kumagai et al. (15) with minor modifications. Briefly, 5 million PBMC isolated by Ficoll-diatrizoate density gradient separation were washed and resuspended in culture media with 10% human plasma. The mononuclear cells were then incubated in 35-mm plastic culture dishes for 1 h at 37°C in 5% CO_2 in humidified air. Nonadherent cells were collected, washed, and resuspended in media for culture. Adherent cells were harvested from the dishes by vigorous scraping with a rubber policeman and collected in a balanced salt solution lacking Ca^{++} and Mg^{++} and supplemented with 0.002 M EDTA. The cells were then washed three times and resuspended in media for culture. The adherent cell population was >50% esterase positive whereas the nonadherent population was <5% esterase positive.

Preparation of Non-T Cells. To define the T helper cell requirement for the generation of PFC, T cell-depleted populations (B cell-and adherent cell-enriched) were prepared. To produce the T cell-depleted population, SRBC treated with 2-amino-ethyliso-thiouronium bromide (AET) (16) were added to PBMC at a ratio of 150:1, AET-SRBC:PBMC, and suspended in a balanced salt solution supplemented with 10% human plasma. The cells were pelleted at 200 *g* for 8 min and incubated at 4°C for 1–2 h. After incubation, the cells were gently resuspended in an equal volume of balanced salt solution, layered over a Ficoll-diatrizoate gradient, and sequentially centrifuged at 400 *g* for 20 min and at 2,250 *g* for 7 min. The population of cells remaining at the interphase was collected, washed, and resuspended in culture media. This cell population contained <3% rosetted cells and a variable percentage of monocytes.

Preparation of T Cells. Because the SRBC rosetting cell population contained some B cells with SRBC receptors, precursors for PFC in this assay, the T cells required for these studies were purified using the technique of rabbit anti-human $(Fab')_2$ cellular immunoabsorbent chromatography to remove surface immunoglobulin-positive cells. The technique of immunoabsorbent chromatography, which has been described previously (17, 18), was modified slightly in this study. Briefly, PBMC isolated by Ficoll-diatrizoate density-gradient centrifugation and suspended in RPMI 1640 media supplemented with 2.5 mM EDTA were applied to a column containing purified rabbit anti-human $(Fab')_2$ fragments covalently conjugated to Sephadex G-200 bead polymers (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The columns before cell application were equilibrated with RPMI 1640–EDTA buffer. The T cell-enriched population was eluted from the column with 15 ml of buffer, washed, and resuspended in media for culture.

Results

Using the basic culture and assay procedure discussed in Materials and Methods, human PBMC could be stimulated by SRBC in the absence of nonspecific polyclonal activators to become cells that synthesize and secrete specific antibody. In the following sections we consider the critical conditions required for the generation of antigen-specific macroscopic PFC, including the definition of such elements of the system as the optimal dose of antigen, length of culture period, concentration of PBMC required, and plasma requirements. In addition, the specificity of the reaction and requirements for T cells and monocytes were determined.

Definition of Antigen Requirements. We studied the effect of different concentrations of SRBC antigen added at the initiation of the culture period by evaluating the number of PFC generated when PBMC from three different normal individuals were exposed to varying concentrations of SRBC in culture. In these experiments, 5×10^6 PBMC were co-cultured for 11 d with various concentrations of antigen ranging from 0 to 10^8 SRBC/culture. All cultures were rocked throughout the period of incubation, and supplemented daily with 75 μl of culture media containing 10% human hypo-

gammaglobulinemic plasma. As shown in Fig. 1, no PFC were generated in the absence of exogenous antigen. Maximal PFC responses were observed with antigen concentrations ranging from 10^6 to 10^7 SRBC/culture. On average, the peak response occurred with 5×10^6 SRBC/culture. Furthermore, antigen concentrations in excess of 10^7 SRBC/culture markedly inhibited or abrogated the PFC response. Based on these observations, the optimal antigen concentration, 5×10^6 SRBC/culture, was used in all further studies.

PFC Generation as a Function of the Length of the Culture Period. In studies reported by others, the maximal PFC response was found to occur between 5 and 8 d of culture in both PWM-activated human cultures assayed for polyclonal immunoglobulin production by the reverse hemolytic plaque assay (19), as well as several human antigen-specific assays previously cited (9–12). Because significant variation in the optimal incubation time varies with the assay, we readdressed this issue in our system. 5 million PBMC from each of three different normal individuals were co-cultured with 5×10^6 SRBC under optimal culture conditions and harvested at various times. No anti-SRBC response was observed until the 5th d of culture (Fig. 2). As the period of incubation was extended beyond day 5, PFC generation was observed. The response peaked between days 9 and 12 of incubation with a rapid fall in this response by day 15. It should be noted that there was individual variation in the day of optimal response. Although no one day represented 100% of the maximal response for all individuals, based on the results in Fig. 2, we have selected an incubation time of 10 or 11 d for all subsequent studies.

The Number of PBMC Required for the Optimal PFC Response. The assays of both Dosch and Gelfand (13) and Delfraissy et al. (12) suggest that a significant PFC

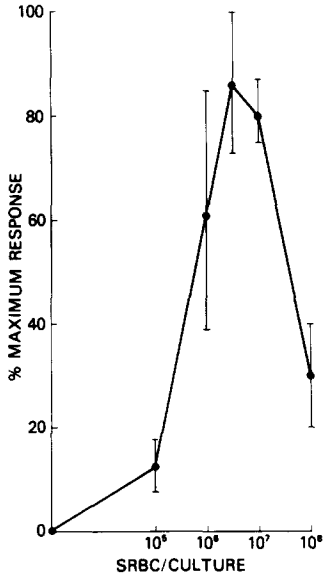

FIG. 1. The effect of varying the antigen concentration on the number of PFC generated. 5 million PBMC from three separate individuals were cultured with various numbers of SRBC. The number of plaques generated at each antigen concentration was normalized to the percent of the maximum response observed for each individual. Each point in the figure represent the mean of the percent maximum response for each of three individuals ± SEM.

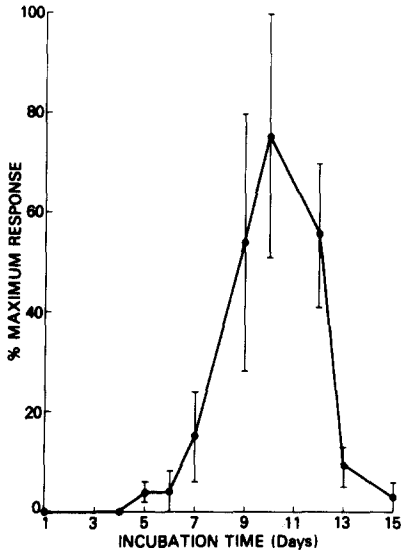

FIG. 2. The time-course of the anti-SRBC response. 5 million PBMC from three separate individuals were cultured with 5 million SRBC and the PFC response assessed on different days. The number of plaques generated at each time period was normalized to the percent of the maximum response observed for each individual. The points on the figure represent the mean of the percent maximum response for all three individuals ± SEM.

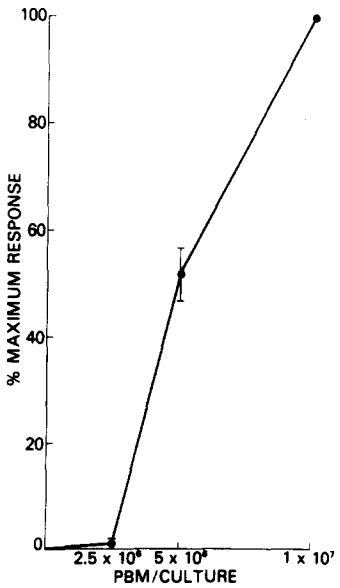

FIG. 3. Plaque-forming cell generation as a function of the number of PBMC cultured. Increasing numbers of PBMC were cultured for 11 d with 5×10^6 SRBC and the PFC response measured. The response at each PBMC concentration is expressed as the percent of the maximum response observed. Each point represents the mean of three replicate cultures ± SEM.

response could require up to 5×10^6–10×10^6 PBMC/culture. Other human antigen-specific assays require far fewer cells (10, 11), and human culture systems using polyclonal stimuli such as PWM require as few as 10^6 PBMC/culture to obtain a PFC response (19). We designed a series of experiments to define the minimum number of cells required to generate a PFC response of significant amplitude and yet remain on the linear portion of the curve relating PFC response to the number of cells cultured. Graded numbers of PBMC ranging from 0 to 10^7 cells from three different normal individuals were cultured with 5×10^6 SRBC under optimal culture conditions. After 11 d of incubation, cells were harvested and the PFC were enumerated. As can be seen in Fig. 3, cultures containing from 0 to 2.5×10^6 PBMC generated few plaques. A sharp increment in the number of PFC is seen with higher cell concentrations, with a linear relationship between cell density and PFC response in the range between 5×10^6 and 5×10^7 PBMC/culture. Based on these observations, we routinely chose to culture 5×10^6 PBMC in 1 ml of media in 35-mm tissue culture dishes.

PFC Response of Normal Individuals. The in vitro culture system was applied to the study of the PBMC from 19 unselected normal individuals. 5 million mononuclear cells from each individual were cultured in triplicate with 5×10^6 SRBC for 10–11 d under the conditions described above. PBMC from all individuals studied generated anti-SRBC plaques at the completion of the culture period. The mean responses of the different individuals (Fig. 4) ranged from 40 to 2,440 plaques per culture (the geometric mean and SEM for all 19 individuals was 326 ×/÷ 1.3 plaques/culture).

Plasma Requirements. Appropriate supporting plasma is critical for in vitro immunoglobulin synthesis. We designed a series of experiments allowing us to evaluate the efficacy of either FCS, or human plasma as the protein source in culture. 5 million

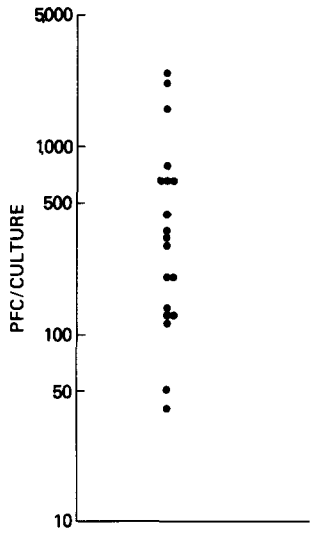

FIG. 4. The number of PFC generated by PBMC from 19 normal individuals. 5 million PBMC from 19 normal individuals were cultured with 5×10^6 SRBC for 10–11 d as indicated in the text, and the number of anti-SRBC PFC generated was enumerated. Each point represents the mean response of triplicate cultures. The geometric mean response and SEM of all 19 individuals was 326 ×/÷ 1.3 plaques/culture.

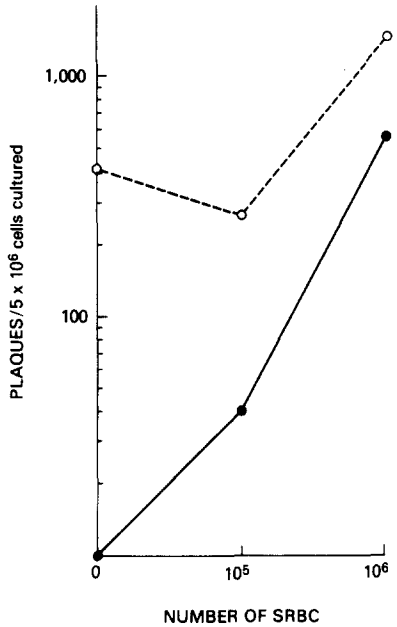

FIG. 5. The effect of absorption of plasma with antigen. Each point represents the anti-SRBC PFC response obtained when 5×10^6 PBMC from one individual were cultured with various numbers of SRBC for 11 d in the presence of 10% human plasma unabsorbed (●) or absorbed (○) with SRBC.

PBMC were co-cultured with 5×10^6 SRBC for 11 d in the presence of either culture media supplemented with 10% heat-inactivated FCS or 10% human plasma collected in preservative-free heparin from a donor with untreated common variable hypogammaglobulinemia. Cells were incubated under optimal conditions. PBMC cultured in the presence of human plasma generated 295 ± 15 plaques. Comparable results were obtained with plasma from four of four patients with common variable immunodeficiency diseases and one of two normal individuals. Plasma from patients with hypogammaglobulinemia was used in all subsequent studies. In contrast to these observations made with cells cultured with human plasma, there was no response observed with cells cultured in the presence of FCS. The addition of 5% FCS to cultures containing 10% human plasma resulted in a 90% inhibition (from 295 ± 15 to 30 ± 5) of PFC generation. Furthermore, 1×10^6 PBMC exposed to FCS for several hours, washed free of the protein, and co-cultured with 4×10^6 PBMC in the presence of 10% human plasma resulted in up to 60% inhibition (observed response 115 ± 50 plaques) of the expected response. Thus, it appears that in this assay human plasma is required for the generation of an antigen-specific response. Furthermore, FCS is not only incapable of supporting a response but also is inhibitory. In addition, PBMC exposed to FCS are capable of suppressing a PFC response when co-cultured with PBMC in the presence of human plasma.

Because human plasma is required, care must be taken to eliminate the possibility of artifactual plaques resulting from passive transfer of specific antibody. Formation of such pseudoplaques as described by Muchmore et al. (20) results from the carry-over into the final assay system of SRBC coated with the antibody derived from the

sera used in culture. To avoid this problem, many groups use serum absorbed with the appropriate antigen before culture. We addressed the effect of preabsorption of the plasma with the antigen used in the present study, SRBC, on plaque formation by co-culturing 5×10^6 PBMC with various concentrations of antigen for 11 d under optimal culture conditions in the presence of 10% human plasma that was either unabsorbed or absorbed. For absorbtion, plasma was incubated three times, for 1 h each time, at 4°C, with washed packed SRBC at a ratio of 1:9, SRBC:plasma, respectively. As can be seen in Fig. 5, at all SRBC concentrations used during the culture period, the PFC response generated in the presence of absorbed plasma was greater than the number generated in the presence of unabsorbed plasma. Furthermore, in the absence of added antigen, absorbed plasma supported a generous PFC response. This is in contrast to the lack of a response by PBMC cultured with unabsorbed plasma in the absence of added antigen. We believe the most likely explanation for these observations is that a concentration of antigen sufficient to generate a PFC response is eluted from the intact erythrocytes during the absorption procedure. This may explain the synthesis of anti-SRBC antibody by PBMC cultured in sera absorbed against SRBC and stimulated by PWM described by Fauci and Pratt (9). Because absorbed plasma may contain unknown concentrations of antigen, we have chosen to use unabsorbed plasma in all cultures. To determine whether the plaques generated by cells stimulated in the presence of unabsorbed hypogammaglobulinemic plasma reflected antibody-synthesizing cells or, alternatively, false plaques resulting from the passive carry-over of antibody, cells were cultured in the presence of cycloheximide, a drug that inhibits de novo antibody synthesis but not false plaque formation. The addition of this drug to cultures 12 h before cell harvest and at the time of plaquing reduced the number of plaques from 501 ± 18 in the control cultures to 40 ± 18 in the treated cultures, a greater than 10-fold reduction in plaque formation. This response to cycloheximide demonstrates the dependence on de novo protein synthesis for the generation of a plaque-forming response, and eliminates the possibility that the response measured by this assay is the result of pseudoplaque formation.

Dependence on Complement for Plaque Development. The absolute dependence on complement in the postculture plaque-forming assay was shown in a series of experiments in which PBMC from four individuals were cultured under optimal conditions and plaqued with and without complement. The PFC response enumerated in the presence of complement ranged from 135 to 630 plaques/culture, whereas, no response was elicited when cells were plaqued in its absence.

Specificity of the Antibody Response. The specificity of the antibody response was assessed by culturing PBMC with antigens and then plaquing against both the immunizing as well as unrelated target cells. 5 million PBMC were cultured with 5×10^6 SRBC or 10^7 HRBC, the antigen concentration found to be optimal for the anti-HRBC response. Sensitized cells were then plaqued against both SRBC and HRBC. As can be seen in Table I, cells sensitized to SRBC generated 290 ± 11 anti-SRBC but no anti-HRBC plaques. Similarly, PBMC sensitized to HRBC generated 110 ± 5 anti-HRBC but only 5 ± 1 anti-SRBC plaques per culture. These results demonstrate the high degree of specificity of the antibody response for the sensitizing antigen. However, we have observed that PBMC sensitized to erythrocytes of more

TABLE I

Hemolytic Plaques Generated by Sensitized PBMC against the Immunizing and an Unrelated Antigen

Sensitizing erythrocytes	Hemolytic plaques	
	Anti-SRBC*	Anti-HRBC*
SRBC	290 ± 11	0
HRBC	5 ± 1	110 ± 5

* The hemolytic plaque response is expressed as the mean number of plaques generated by three replicate cultures ± SEM.

TABLE II

Lack of Polyclonal Stimulation by SRBC

Sensitizing cells	Anti-SRBC-specific plaques*	Reverse plaques (developed with an anti-IgM antibody)*‡
None	0	688 ± 64
SRBC	32 ± 7	922 ± 112

* 5 million PBMC were cultured for 11 d in the presence or absence of antigen (SRBC). The number of antigen-specific and the number of reverse anti-IgM plaques generated by each culture were enumerated. Each value represents the mean of three replicate cultures expressed as the number of plaques per 10^6 cells cultured ± SEM.

‡ The polyclonal IgM response was assessed by enumerating the IgM-secreting cells by a reverse plaque technique developed with an anti-IgM antibody. The difference in the number of anti-IgM reverse plaques from cultures with and without antigen is not statistically significant ($P > 0.2$).

closely related species (i.e., sheep and bovine) generate a PFC response with considerable cross-reactivity (data not shown).

Nonpolyclonal Nature of the Response to the SRBC Antigen. A major shortcoming of several of the presently available human culture systems used for the generation of antigen-specific PFC is the presence of polyclonal activators in the cultures (9, 10, 12). No known polyclonal activators were used in the assay described here. However, one cannot preclude the possibility that the antigen itself (SRBC) may have polyclonal stimulatory properties. We addressed this possibility with a series of experiments in which PBMC were co-cultured with either no antigen or with 5×10^6 SRBC. After 11 d of incubation, the cells were harvested and plaqued against either SRBC alone, or staphlococcal protein A-bound SRBC plus an anti-IgM developing reagent. Those assays that include staphylococcal protein A-treated SRBC and anti-IgM would enumerate all cells producing IgM, not solely those producing anti-SRBC antibody, and are thus a measure of polyclonal immunoglobulin production. The results are shown in Table II. PBMC that were not exposed to antigen generated no antigen-specific plaques, whereas when evaluated in a modified reverse hemolytic plaque assay, the same cells generated 688 ± 64 anti-IgM plaques/10^6 cells cultured. Alternatively, PBMC exposed to antigen generated 32 ± 7 antigen-specific plaques/10^6 cells cultured, whereas in the reverse hemolytic plaque assay, 922 ± 112 plaques were generated. Because the number of reverse plaques generated with and without antigen are not significantly different ($P > 0.2$), one can conclude that SRBC do not possess significant polyclonal stimulatory properties.

Requirements for Adherent Cells and T Cells for the Antibody Response. The requirement for adherent cells for the antibody response in this culture system was determined using cultures of cells depleted of adherent cells and cultures of adherent cell-depleted populations that were reconstituted with graded numbers of adherent cells. 5 million PBMC depleted of plastic adherent cells were cultured under optimal conditions with SRBC. Similarly, 4×10^6 nonadherent cells were cultured alone and with graded numbers of adherent cells. The results are shown in Fig. 6. Nonadherent cell-depleted populations generated 20 ± 5 PFC/culture, <10% of that generated by unseparated cells. The readdition of 2,000-rad irradiated adherent cells so that they constituted an additional 5% of the total cell population reconstituted the normal response. However, further addition of irradiated adherent cells so that they constituted an additional 15% of the total population resulted in suppression of the expected PFC response to <5% of the peak response.

Demonstration of T cell dependency required the modifications of the routine methods for obtaining T and non-T cell populations that are indicated in Materials and Methods. Non-T cells eluted with gamma globulin from an anti-$(Fab')_2$ column were found to be suppressive when co-cultured with unseparated cells (data not shown). Furthermore, T cells purified by AET-SRBC rosetting techniques were consistently contaminated by cells with anti-SRBC PFC capacity. This later observation is most likely caused by a population of B cells with SRBC surface receptors that co-purify with the rosetted T cells. To circumvent these problems, we co-cultured T cells derived from mononuclear cells that did not adhere to an anti$(FAB')_2$ column (>95% T cells, as assessed by rosetting with SRBC), and non-T cells obtained from cells that did not form rosettes with AET-SRBC. The results of these studies are illustrated in Table III. Neither T cells nor non-T cells alone were capable of generating a PFC response. However, activity was restored by co-culturing 4×10^6 T cells with 10^6 non-T cells. It should be noted that in this system, 2,000-rad irradiated

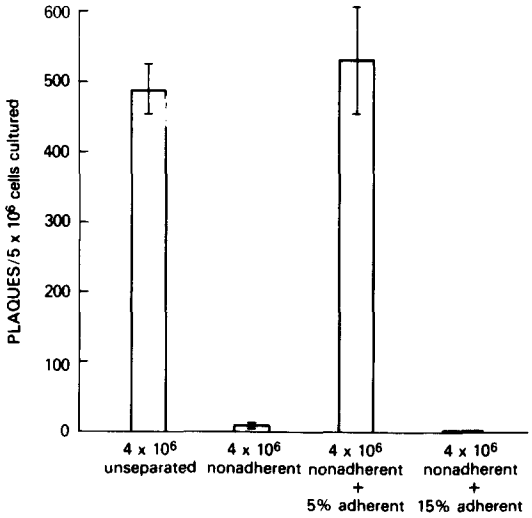

FIG. 6. Requirements for adherent cells. PBMC were cultured unseparated, after the removal of plastic adherent cells and after the readdition of an additional 5 or 15% plastic adherent cells. Plastic adherent cells were treated with 2,000 rad irradiation before their addition to the cultures. Each bar represents the mean response of triplicate cultures ± SEM.

TABLE III

The Anti-SRBC Response Is T Cell Dependent

Cells cultured*	PFC response/culture
5×10^6 unseparated cells	40 ± 8
5×10^6 non-T cells	0
5×10^6 T cells	0
1×10^6 non-T cells + 4×10^6 T cells	69 ± 29
1×10^6 non-T cells‡ + 4×10^6 T cells	0
1×10^6 non-T cells + 4×10^6 T cells‡	0

* Non-T cells were negatively selected by AET-SRBC rosetting. T cells were negatively selected by anti-$(Fab')_2$ immunoabsorbent chromatography. Cells treated with 2,000 rad irradiation are indicated by double dagger (‡). The number of PFC recorded represents the mean of triplicate cultures ± SEM.

T cells are not capable of providing sufficient help to generate an antibody response. This suggests that one or more of the T cells required to provide antigen-specific T cell help is radiosensitive to 2,000 rad. The alternative possibility that the T cell population was contaminated by B cells and that the non-T cell population was providing a radioresistant accessory cell necessary for those B cells to synthesize antibody was excluded by the observation that synthesis could not be demonstrated by co-cultures of T cells and irradiated (2,000 rad) non-T cells.

Based on these observations, we concluded that the human in vitro anti-SRBC response generated in this culture and assay system is dependent on both T cells and adherent cells, and furthermore, at least one subpopulation of T cells required for antigen-specific antibody production is sensitive to 2,000 rad irradiation.

Discussion

The potential importance of an in vitro antigen-specific PFC culture and assay system to the study of the mechanisms of human peripheral B cell activation can be inferred from the vast contribution such a technique has made to our understanding of the cellular interactions associated with murine spleen cell activation by antigen. Unfortunately, attempts at establishing a reproducible culture system for a specific antibody response that requires no polyclonal activators and that is applicable to humans and, in particular, human PBMC, have generally not met with success. In this paper, we have described culture conditions suitable for the in vitro induction of an antigen-specific primary antibody response by human PBMC, and a technique by which this response can be reproducibly quantitated. This assay has several advantages over previously described human antigen-specific systems. The culture techniques described by Fauci and Pratt (9), Luzatti et al. (10), Delfraissy et al. (12), and Hoffmann (11), require the addition of polyclonal activators (PWM, Epstein-Barr virus, haptenated carriers possessing polyclonal stimulatory capacity [polyacrylamide beads]), or proliferative stimuli (heat-inactivated *S. aureus*). The culture system detailed in this paper is solely dependent on an antigen (SRBC) that itself possesses no demonstrable nonspecific stimulatory activity. Other reported assay techniques such as that described by Dosch and Gelfand (13), although requiring no stimulus other than antigen, involve a methodology for the development of plaques that is technically difficult to reproduce and results in the generation of microscopic plaques. Furthermore, the time available for the generation of plaques and their subsequent enumer-

ation is restricted by the relatively rapid deterioration of the erythrocyte monolayers constructed by this technique. We have found that fixation of the target erythrocytes and the sensitized mononuclear cells in an agarose matrix with complement results in monolayers that are stable over time allowing for a sufficient incubation period for the generation of large, macroscopic plaques.

The success of this system can be attributed to several critical factors. Optimal cell density is essential. Large numbers of PBMC must be cultured in order to generate a response. Because the number of antigen-specific B and T lymphocyte precursors are probably in low frequency in the nonimmunized host, it is not surprising that large numbers of cells per culture would be required to insure an appropriate repertoire of cells for an antigen specific response. Prolonging the incubation time to 10–12 d, continuous rocking during the entire period of incubation, culturing in large flat-bottomed culture dishes in the presence of human plasma, daily supplementation with culture media and plasma, and appropriate antigen concentration are all vital to the generation of antigen-specific PFC. Furthermore, plaquing sensitized PBMC in target cell monolayers fixed in an agarose matrix with the appropriate concentration of antigen for incubation periods of 2–3 h is essential for the formation of large hemolytic plaques.

It is imperative that the plaques generated in an assay be shown to represent antibody production and not the formation of false plaques. Absorbed plasma was not routinely used in our assays. However, we are confident that plaque formation is the product of de novo protein synthesis because plaque formation was inhibited by the addition of cycloheximide to the culture system.

This culture system can be used to study the cellular interactions and regulatory mechanisms involved in human antigen-specific antibody production in vitro because it can be demonstrated that the response to SRBC in this system is dependent on populations of both T cells and plastic-adherent cells. Of particular interest is the finding that helper T cell function in this response is sensitive to 2,000 rad irradiation. Thomas et al. (21) have shown that antibody production by PWM-stimulated peripheral blood B cells is augemented by prior irradiation of the T cells when cultured at high T:B cell ratios, whereas, antibody production is reduced by such irradiation at low T:B cell ratios. Lipsky (22) observed a similar apparent radiosensitivity of the T cell help required for the in vitro staphlococcal protein A system when examined with low T:B cell ratios. These observations suggest the presence of both a radiosensitive and radioresistant helper T cell population. We have further suggested that the two T helper cell populations differing in terms of their radiosensitivity can be differentiated in terms of their reactivity with an antibody that reacts with activated T cells termed anti-Tac antibody (23). Distinct populations of helper T cells that can act independently and synergistically in in vitro antigen-specific antibody production have been identified in the murine system (24–28). Marrack and Kappler (24), and Tada et al. (26), defined both on Ia^+ antigen-nonspecific and an Ia^- antigen-specific helper T cell population that could be further separated on the basis of nylon wool adherence. Two independent murine splenic helper T cell populations capable of functioning synergistically and characterized by differences in both radiosensitivity and nylon wool adherance have been described by Agarossi et al. (28). We have shown that the human antigen-specific anti-SRBC response elicited by the technique described in this paper is dependent on at least one radiosensitive

helper T cell population. Whether other T cell subsets are required for the full expression of this response has yet to be determined.

Summary

We have described a culture and assay system for the sensitization of human peripheral blood mononuclear cells with a T cell-dependent antigen, sheep erythrocytes, in the absence of nonspecific stimulatory agents and with the subsequent generation of macroscopic hemolytic plaques. We have shown that the antibody produced by the plaque-forming cells generated in this cuture system is specific for the sensitizing antigen, and that the plaques created are not false plaques because their formation is inhibited by cycloheximide. The success of this system can be attributed to several critical factors including large numbers of peripheral blood mononuclear cells (5×10^6/culture), a prolonged period of incubation (10–11 d), continuous rocking during the entire period of incubation, culturing in large (35-mm) flat-bottomed culture dishes in the presence of human plasma, and the appropriate antigen concentration (5×10^6 sheep erythrocytes/culture). Furthermore, the generation of macroscopic hemolytic plaques requires plaquing sensitized peripheral blood mononuclear cells in target cell monolayers fixed in an agarose matrix with an incubation period of 2–3 h. We have further shown that the antigen-specific response measured by this system is dependent on adherent cells and T lymphocytes. At least one population of the helper T cells is sensitive to 2,000 rad irradiation. This system is simple, sensitive, and should serve as an effective tool for the analysis of cellular interactions involved in the generation of human antigen-specific plaque-forming cells, the genetic control the human immune response, and the pathophysiology of altered immunoregulation in disease.

Received for publication 1 June 1981.

References

1. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. *Science (Wash. D. C.).* **140:**405.
2. Cunningham, A. J. 1965. A method of increased sensitivity for detecting single antibody-forming cells. *Nature (Lond.).* **207:**1106.
3. Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology.* **14:**599.
4. Mishell, R. I., and R. W. Dutton. 1966. Immunization of normal mouse spleen cell suspensions *in vitro. Science (Wash. D. C.).* **153:**1004.
5. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126:**423.
6. Hoffman, M. K., D. Schmidt, and H. F. Oettgen. 1973. Production of antibody to sheep red blood cells by human tonsil cells *in vitro. Nature (Lond.).* **243:**408.
7. Watanabe, T., K. Yoshizaki, T. Yagura, and Y. Yamamura. 1974. *In vitro* antibody formation by human tonsil lymphocytes. *J. Immunol.* **113:**608.
8. Fauci, A. S., and K. R. Pratt. 1976. Activation of human B lymphocytes. I. Direct plaque-forming cell assay for the measurement of polyclonal activation and antigenic stimulation of human B lymphocytes. *J. Exp. Med.* **144:**674.
9. Fauci, A. S., and K. R. Pratt. 1976. Polyclonal activation of bone-marrow-derived lym-

phocytes from human peripheral blood measured by a direct plaque forming cell assay. *Proc. Natl. Acad. Sci. U. S. A.* **73:**3676.

10. Luzzati, A. L., H. Hengartner, and M. H. Schreier. 1977. Induction of plaque-forming cells in cultured human lymphocytes by combined action of antigen and EB virus. *Nature (Lond.).* **269:**419.
11. Hoffman, M. K. 1980. Antigen-specific induction and regulation of antibody synthesis in cultures of human peripheral blood mononuclear cells. *Proc. Natl. Acad. Sci. U. S. A.* **77:** 1139.
12. Delfraissy, J. F., P. Galanaud, J. Dormont, and C. Wallon. 1977. Primary *in vitro* antibody response from human peripheral blood lymphocytes. *J. Immunol.* **118:**630.
13. Dosch, H. M., and E. W. Gelfand. 1977. Generation of human plaque-forming cells in culture: tissue distribution, antigenic and cellular requirements. *J. Immunol.* **118:**302.
14. Gronowicz, E., A. Coutinho, and F. Melchers. 1976. A plaque assay for all cells secreting Ig of a given type or class. *Eur. J. Immunol.* **6:**58.
15. Kumagai, T., K. Itoh, S. Hinuma, and M. Tada. 1979. Pretreatment of plastic petri dishes with fetal calf serum. A simple method for macrophage isolation. *J. Immunol. Methods.* **29:** 17.
16. Pellegrino, M. A., S. Ferrone, M. P. Dietrich, and R. A. Reisfeld. 1975. Enhancement of sheep red blood cell human lymphocyte rosette formation by the sulfhydryl compound 2-amino-ethyliso-thiouronium. *Clin. Immunol. Immunopathol.* **3:**324.
17. Chess, L., R. P. MacDermott, and S. F. Schlossman. 1974. Immunologic functions of isolated human lymphocyte subpopulations. I. Quantitative isolation of human T and B cells and response to mitogens. *J. Immunol.* **113:**1113.
18. Broder, S., R. L. Edelson, M. A. Lutzner, D. L. Nelson, R. P. MacDermott, M. E. Durm, D. K. Goldman, B. D. Meade, and T. A. Waldmann. 1976. The Sezary syndrome. A malignant proliferation of helper T cells. *J. Clin. Invest.* **58:**1297.
19. Ginsburg, W. W., F. D. Finkelman, and P. E. Lipsky. 1978. Circulating and mitogen induced immunoglobulin secreting cells in human peripheral blood: evaluation by a modified reverse hemolytic plaque assay. *J. Immunol.* **120:**33.
20. Muchmore, A. V., I. Koski, N. Dooley, and R. M. Blaese. 1976. Artifactual plaque formation *in vitro* and *in vivo* due to passive transfer of specific antibody. *J. Immunol.* **116:** 1016.
21. Thomas, Y., J. Sosman, O. Irigoyen, S. M. Friedman, P. C. Kung, G. Goldstein, and L. Chess. 1980. Functional analysis of human T cell subsets defined by monoclonal antibodies. I. Collaborative T-T interactions in the immunoregulation of B cell differentiation. *J. Immunol.* **125:**2402.
22. Lipsky, P. E. 1980. Staphylococcal protein A, a T cell-regulated polyclonal activator of human B cells. *J. Immunol.* **125:**155.
23. Uchiyama, T., D. L. Nelson, T. A. Fleisher, and T. A. Waldmann. 1981. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. II. Expression of Tac antigen on activated cytotoxic killer T cells, suppressor cells, and on one of two types of helper T cells. *J. Immunol.* **126:**1398.
24. Marrack, P. C., and J. W. Kappler. 1975. Antigen-specific and non-specific mediators of T cell/B cell cooperation. I. Evidence for their production by different T cells. *J. Immunol.* **114:**1116.
25. Keller, D. M., J. E. Swierkosz, P. Marrack, and J. W. Kappler. 1980. Two types of functionally distinct, synergizing helper T cells. *J. Immunol.* **124:**1350.
26. Tada, T., T. Takemori, K. Okumura, M. Nonaka, and T. Tokuhisa. 1978. Two distinct

types of helper T cells involved in the secondary antibody response: independent and synergistic effects of Ia^- and Ia^+ helper T cells. *J. Exp. Med.* **147:**446.

27. Janeway, C. A. Jr., R. A. Murgita, F. I. Weinbaum, W. R. Asofsky, and H. Wigzell. 1977. Evidence for an immunoglobulin-dependent antigen-specific helper T cell. *Proc. Natl. Acad. Sci. U. S. A.* **74:**4582.
28. Agarossi, G., C, Mancini, and G. Doria. 1981. Synergism between two helper cell subpopulations characterized by different radiosensitivity and nylon adherence. *J. Immunol.* **126:** 955.