IN VITRO ANTIGEN-INDUCED, ANTIGEN-SPECIFIC ANTIBODY PRODUCTION IN MAN

Specific and Polyclonal Components, Kinetics, and Cellular Requirements

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The availability of an antigen-induced, antigen-specific system to measure in vitro antibody responses by human B cells is critical for the precise delineation of the physiology of human B cell activation and immunoregulation. Specific antibody production has been studied in man for several years. However, in doing so, some of the systems that have been employed have required induction by polyclonal B cell activators, whereas others, which have utilized direct stimulation by antigen, have been technically difficult to perform or unpredictable in terms of individual responses (1–3).

We have previously reported (4) that the unfractionated peripheral blood mononuclear cells from normal human subjects immunized to the soluble protein antigen keyhole limpet hemocyanin (KLH)[1] could be induced in vitro by antigen alone or by pokeweed mitogen (PWM) to produce specific antibody. In each case, the specific antibody production was nonetheless associated with polyclonal activation. In the present study, we have characterized the overlap between antigen specificity and polyclonality in the induction and expression of antigen-induced antibody production and have demonstrated a dichotomy between antigen-specific and polyclonal responses to two distinct protein antigens, KLH and tetanus toxoid (TT). In addition, we have precisely defined the culture conditions and cellular requirements for antigen-induced, antigen-specific antibody production by human peripheral blood B cells.

Materials and Methods

Immunizations. Normal subjects, aged 18–40 yr, received two subcutaneous injections of 5 mg KLH (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif.), which had been dialyzed against phosphate-buffered saline (PBS) and passed through a 0.45-μm millipore filter. Injections were spaced by 2 wk. TT (Wyeth Laboratories, Marietta, Pa.), 5 lf, was also administered subcutaneously to some subjects. Immunizations were carried out under peer-reviewed National Institutes of Health protocols.

Cell Separations. Peripheral blood mononuclear cells were obtained from Hypaque-Ficoll gradients in a standard fashion (5). These unfractionated mononuclear cells were 10–30% monocytes, 70–90% lymphocytes, and 0–5% basophils and granulocytes by morphology and

[1] *Abbreviations used in this paper:* KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; PWM, pokeweed mitogen; TT, tetanus toxoid.

nonspecific esterase staining. Within the lymphocyte population there were 5–10% B cells as determined by surface immunoglobulin (Ig) staining, using a fluorescein-conjugated $F(ab')_2$ fragment goat anti-human Ig (N. L. Cappel Laboratories Inc., Cochranville, Pa.) (6).

T cell-enriched populations were obtained from nylon wool columns (7). Cells eluted from the column were <1% surface Ig positive. T cell depletion was accomplished through the use of the anti-T cell hybridoma antibody Leu-1 (Becton, Dickinson & Co., Oxnard, Calif.) and rabbit serum (Dutchland Laboratories Inc., Dutchland Laboratory Animals Inc., Denver, Pa.) (8). Cells were suspended at a concentration of 10^7/ml in RPMI 1640 (Flow Laboratories, Inc., Rockville, Md.). 1 μg of antibody was added per 5×10^6 cells, and the resulting mixture was combined with pooled, young rabbit serum, which had been screened for cytotoxicity, to yield a final concentration of rabbit serum of 20–30%. The mixture was incubated for 1 h at 37°C and the cells washed three times in RPMI 1640. Approximately 20% of the initial cells remained. These cells were 90–95% viable by trypan blue dye exclusion, 15–40% B cells, 5–10% sheep erythrocyte rosette positive, and 30–50% monocytes by nonspecific esterase staining and morphology.

Monocyte depletion was performed by plate adherence of 40×10^6 unfractionated cells in 2 ml RPMI 1640 containing 1% human A serum for 1 h at 37°C in 100-mm petri dishes (9). The nonadherent cells were then passed through Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, N. J.) (10). Approximately 50% of the initial unfractionated cells remained after these two procedures, and these were <3% monocytes. Monocytes were obtained from continuous density gradients of Percoll (Pharmacia Fine Chemicals) (11) and then treated with 3,000 rad. The purity of the monocyte suspensions was 70–90%.

Culture Conditions. Cultures for the measurement of supernate Ig production were performed in 1 ml of RPMI 1640 containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) in either 12- × 75-mm round-bottomed test tubes or 24-well flat-bottomed plates with 16-mm wells (Costar Data Packaging, Cambridge, Mass.), as indicated. These cultures contained various amounts of KLH, PBS-dialyzed TT (Lederle, Pearl River, N.Y.), or PWM (Grand Island Biological Co.). KLH and TT were diluted in RPMI 1640 containing 10% fetal calf serum. Cultures were incubated in a 5% CO_2 atmosphere at 37°C and rocked at 4 cycle/min. Cultures containing TT were washed free of antigen at day 4 of culture. Cell density in culture was varied as indicated depending on the experiment. Cultures were harvested at day 12 unless otherwise indicated, and supernates were decanted and stored at 4°C until assay, usually within 1 wk.

Cultures for measuring blast transformation were performed as previously described in 96-well microtiter dishes (Limbro Chemical Co., Hamden, Conn.) with 1×10^5 cells/well (12). Cultures contained 0.2 ml RPMI 1640 with 15% human A serum. Various amounts of KLH diluted in 15% human A serum or PWM were added to cultures that were then incubated in a 5% CO_2 atmosphere at 37°C for 5 d.

Assays. Specific and total Ig production were measured using enzyme-linked immunosorbent assays as has been previously reported (4, 13). Flat-bottomed, 96-well microtiter plates (Dynatech Laboratories Inc., Dynatech Corp., Alexandria, Va.) were first coated with 0.2 ml of a carbonate buffer, pH 9.6, containing one of the following proteins: 1 μg/ml KLH, 1 lf/ml TT (Wyeth), 10 μg/ml Fab fragment goat anti-human IgG (Fab fragment; N. L. Cappel Laboratories Inc.), or 10 μg/ml IgG fraction goat anti-human IgM (heavy-chain specific; N. L. Cappel Laboratories Inc.) and allowed to incubate overnight at 4°C. Plates were then washed three times with PBS containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo). Samples were appropriately diluted, added to duplicate wells in a volume of 0.2 ml, and incubated at room temperature for 2 h. The plates were then washed as before, and 0.2 ml of a 1:1,000 dilution of heavy-chain-specific goat anti-human IgG or IgM conjugated to alkaline phosphatase (Sigma Chemical Co.) was added to each well. The plates were incubated overnight at 4°C, washed again, and then allowed to react with 0.2 ml per well of a 1 mg/ml solution of *p*-nitrophenyl phosphate (Sigma Chemical Co.) in a pH 8.6 carbonate buffer containing 0.001 M $MgCl_2$. The subsequent development of color because of enzymatic conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol was measured using a multichannel spectrophotometer (Flow Laboratories, Inc.) and related to that seen with a standard serum containing a known amount of anti-TT antibody expressed in AU per ml, a pool of human

sera with a known amount of IgG or IgM (N. L. Cappel Laboratories Inc.), or a standard sera rich in either anti-KLH IgM or IgG. One DT_M unit was defined as the amount of KLH-specific IgM present in a 1:10,000 dilution, and one DT_G was defined as the amount of KLH-specific IgG present in a 1:100,000 dilution of the appropriate reference serum.

Blast transformation was measured in cultures set up as outlined above. Cultures were pulsed with 2 μCi of [^{3}H]thymidine on day 5 and harvested 4 h later on a Titertek cell harvester (Flow Laboratories, Inc.). The filter disks were placed in scintillation vials with 3 ml Aquasol scintillation fluid (New England Nuclear, Boston, Mass.) and counted in a scintillation counter (model LS-350, Beckman Instruments, Fullerton, Calif).

Results

In Vitro Induction of KLH-specific IgM Antibody Responses. KLH-specific IgM antibody production was triggered by concentrations of KLH as low as 1 ng/ml in immunized subjects (Fig. 1). Peak responses were seen at concentrations of 50–200 ng KLH/ml. The levels of in vitro specific antibody production began to decline at in vitro antigen concentrations in the range of 1 μg/ml and were totally suppressed at concentrations of 20 μg/ml. This has been a consistent finding in all 20 subjects studied. Of note is the fact that none of the nonimmunized subjects studied have been able to make specific antibody in response to in vitro KLH (Fig. 1).

Similar curves were generated when the cells were washed free of antigen at day 4

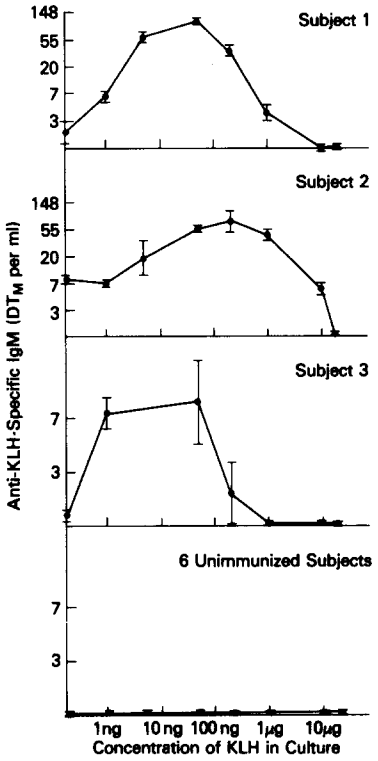

FIG. 1. In vitro induction of KLH-specific IgM antibody responses as a function of in vitro antigen concentration. The responses of three individual immunized subjects and of a pool of six nonimmunized subjects are shown. Cultures were performed in flat-bottomed vessels with 1.5×10^6 cells per 1 ml culture. Data are expressed as geometric means $\overset{\times}{\div}$ SEM for triplicate cultures.

TABLE I

*In Vitro Anti-KLH-specific Antibody Production**

Subject	Round-bottomed culture vessels‡			Flat-bottomed culture vessels§		
	Control	Antigen alone‖	PWM alone	Control	Antigen alone	PWM alone
1	1	12	1			
2	6	358	302			
3	3	56	160			
4	1	16	200			
5	1	14	2			
6	1	7	6			
7	4	210	141			
8	1	1	1			
9	4	100	6			
10	1	75	58			
11	$2 \overset{\times}{\div} 2.3$¶	$72 \overset{\times}{\div} 1.5$	$42 \overset{\times}{\div} 1.5$			
12	$48 \overset{\times}{\div} 1.2$	$1983 \overset{\times}{\div} 1.2$	$1277 \overset{\times}{\div} 1.1$			
13	$15 \overset{\times}{\div} 1.1$	$126 \overset{\times}{\div} 1.5$	$11 \overset{\times}{\div} 2.4$			
14	$16 \overset{\times}{\div} 1.5$	$18 \overset{\times}{\div} 1.4$	$51 \overset{\times}{\div} 1.1$			
15	1	$282 \overset{\times}{\div} 1.2$	$65 \overset{\times}{\div} 1.1$			
16	$1 \overset{\times}{\div} 1.1$	$49 \overset{\times}{\div} 1.3$	$110 \overset{\times}{\div} 1.1$	$2 \overset{\times}{\div} 1.5$	$109 \overset{\times}{\div} 1.1$	$1 \overset{\times}{\div} 1.1$
17	1	$3 \overset{\times}{\div} 2.5$	$50 \overset{\times}{\div} 2.0$	$1 \overset{\times}{\div} 1.2$	$15 \overset{\times}{\div} 1.1$	1
18	$3 \overset{\times}{\div} 1.3$	$123 \overset{\times}{\div} 1.1$	$54 \overset{\times}{\div} 2.4$	$9 \overset{\times}{\div} 1.1$	$72 \overset{\times}{\div} 1.5$	$79 \overset{\times}{\div} 1.1$
19	$11 \overset{\times}{\div} 3.4$	$39 \overset{\times}{\div} 1.9$	$50 \overset{\times}{\div} 2.0$	1	$6 \overset{\times}{\div} 1.4$	$4 \overset{\times}{\div} 1.5$
20	1	$120 \overset{\times}{\div} 1.2$	$32 \overset{\times}{\div} 1.5$	1	$11 \overset{\times}{\div} 3.3$	1
Average (1–20)	$3 \overset{\times}{\div} 1.3$	$48 \overset{\times}{\div} 1.5$	$34 \overset{\times}{\div} 1.5$			
Average (16–20)¶	$2 \overset{\times}{\div} 1.6$	$38 \overset{\times}{\div} 1.9$	$54 \overset{\times}{\div} 1.2$	$2 \overset{\times}{\div} 1.5$	$24 \overset{\times}{\div} 1.7$	$3 \overset{\times}{\div} 2.3$

* Data are expressed as DT_M U/ml.

‡ 5×10^6 cells in 1 ml.

§ 1.5×10^6 cells in 1 ml.

‖ 200 ng KLH.

¶ Geometric mean $\overset{\times}{\div}$ SEM for triplicate cultures.

Cultures of subjects 16–20 were simultaneously performed in round-bottomed and flat-bottomed vessels, and the responses under both conditions were compared.

and recultured in fresh media (4). This eliminated the possibility of a significant assay artifact related to the antigen in culture.

The peak in vitro KLH-specific, IgM antibody responses of the unfractionated mononuclear cells from 20 consecutive subjects immunized to KLH are listed in Table I. All but two subjects (8 and 14) showed at least a sixfold rise in specific in vitro antibody production in response to KLH. The average increase was 16-fold. One subject who failed to respond (subject 8) was experiencing a viral syndrome at the time of the study.

PWM was also capable of triggering antigen-specific antibody production as a part of the total polyclonal response induced by the mitogen. In this regard, antigen-induced and PWM-induced antigen-specific responses were usually comparable in magnitude in the cultures performed in the round-bottomed vessels. However, some subjects (1, 9, and 13) manifested a much greater anti-KLH response to antigenic

stimulation than to PWM stimulation, whereas other subjects (4 and 17) responded better to PWM. It is of particular importance to point out that the magnitude of the PWM-induced specific antibody response was strictly dependent upon the culture conditions. In four out of the five subjects whose lymphocytes were cultured in flat-bottomed as well as round-bottomed vessels, at high and low cell densities, PWM-induced specific antibody synthesis was apparent only in the cultures done in the round-bottomed vessels at the lower cell number. This phenomenon will be discussed in detail in a later section.

In Vivo Kinetics of Antigen-specific Antibody Production. Maximum in vitro antigen-inducible, antigen-specific IgM antibody responses were obtained 2 wk after booster immunization (Fig. 2). This same pattern of appearance of responsiveness was seen in all five subjects studied in this manner. However, the duration of the expression of in vitro responsiveness to antigenic stimulation was highly variable. Unfractionated mononuclear cells from some subjects became unresponsive to antigen as early as 4 wk after booster immunization, whereas the cells from others could be triggered by antigen to produce specific IgM antibody responses up to 1.5 yr after booster immunization. Thus, the kinetics of the in vivo induction of cells that could be triggered in vitro was consistent from subject to subject, whereas the duration in the peripheral circulation of this antigen-responsive population of cells was quite variable.

In contrast to the consistency seen in the development of antigen-induced antigen-

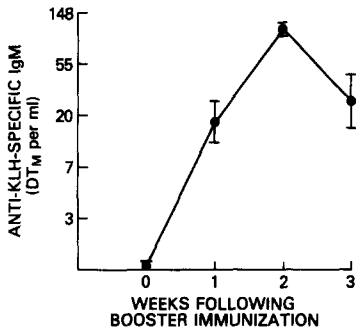

FIG. 2. In vivo kinetics of in vitro antigen-induced antibody synthesis. Cells were obtained from a KLH-immune individual at the times indicated and stimulated in vitro with 200 ng KLH. Cultures were performed in round-bottomed vessels with 5×10^5 cells/1 ml culture. Data are expressed as geometric means $\overset{\times}{+}$ SEM for triplicate cultures.

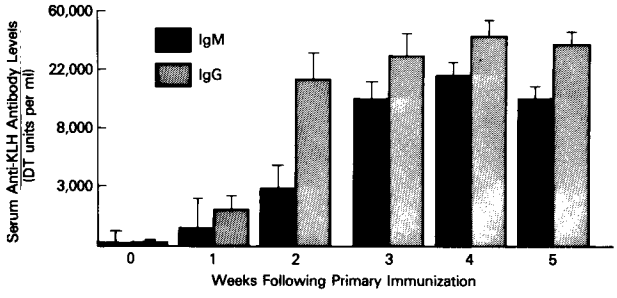

FIG. 3. Serum anti-KLH antibody levels as a function of time after immunization. Blood samples from six subjects were obtained at the intervals indicated and assayed for IgG and IgM antibodies directed against KLH. Data are expressed as geometric means $\overset{\times}{+}$ SEM for the six subjects.

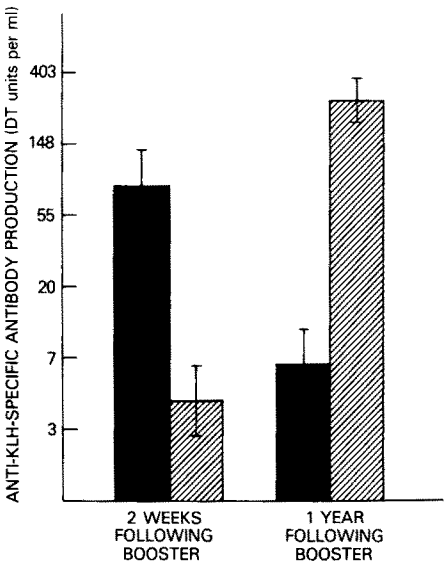

FIG. 4. Isotype expression of PWM-induced specific anti-KLH antibody production. Four subjects were studied at the intervals shown. Cultures were done in round-bottomed vessels with 5×10^6 cells per 1-ml cultures. Data are expressed as geometric means ⁄ SEM for the four subjects. ■, IgM; ▨, IgG.

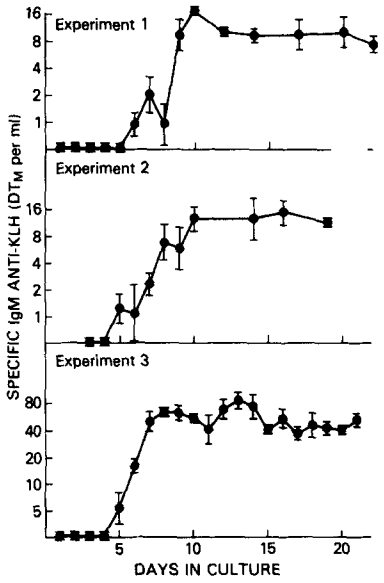

FIG. 5. In vitro kinetics of antigen-induced, antigen-specific antibody production for three individual subjects. Triplicate cultures performed in flat-bottomed vessels with 1.5×10^6 cells/1 ml culture and 200 ng KLH were harvested on the days shown. Data are expressed as geometric means ⁄ SEM for the triplicate cultures.

specific IgM responses, the antigen-specific IgG responses were highly variable. Unfractionated mononuclear cells of some subjects produced specific IgG antibody as early as 1 wk after booster immunization whereas the cells of others were unable to be

triggered by antigen to produce specific IgG antibody as late as 1 yr after booster immunization. This occurred despite the persistent ability of those cells to produce specific IgM in response to antigen and the presence of substantial amounts of specific IgG antibody in the serum (Fig. 3).

The PWM-triggered anti-KLH antibody responses obtained several weeks after booster immunization were predominantly of the IgM isotype (Fig. 4), despite the simultaneous presence of high levels of KLH-specific IgG and IgM antibodies in serum (Fig. 3). In contrast, the unfractionated mononuclear cells of these same individuals, triggered 1 yr later by PWM produced in vitro anti-KLH antibody responses primarily of the IgG isotype (Fig. 4).

In Vitro Kinetics. Specific antibody production was first detected at day 4 or 5 of culture, with levels increasing to a maximum by day 10 (Fig. 5). The kinetics of specific anti-KLH IgG, total IgM, and total IgG production were identical with those of specific anti-KLH IgM production. Of note is the fact that antibody was not detected in any of the culture supernates collected from days 0 through 3. Thus, antibody production was de novo, and it was necessary to incubate cultures for at least 10 d in order to obtain maximal responses. Prior irradiation of the cells with 5,000 rad before culture completely eliminated in vitro antibody production, confirming the de novo nature of the response (4).

Relationship between Specific and Polyclonal Responses. Cell density was a major factor influencing the magnitude of the in vitro specific antibody response to antigen alone or the polyclonal B cell activator PWM. In flat-bottomed vessels, PWM induction of specific antibody production was maximum at a cell density of 5×10^5 cells/well, whereas antigen-induced specific antibody production was optimum at 1.5×10^6 cells/well (Fig. 6). Of note is the fact that PWM-induced specific antibody synthesis was markedly reduced at the cell density yielding the maximal specific response to antigen. The converse was also true, that is, antigen-induced specific responses were diminished at the cell density that yielded the maximal PWM response.

The geometry of the culture vessel was critical in determining the specificity of the

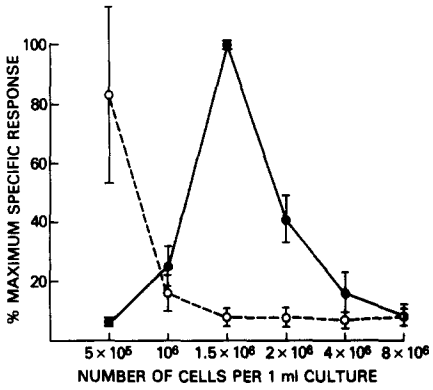

FIG. 6. Role of cell density in influencing the magnitude of PWM or antigen-induced specific antibody production. Triplicate cultures were performed on the unfractionated mononuclear cells from three subjects in flat-bottomed vessels at various cell densities and stimulated with either 200 ng KLH (●) or a 1:200 dilution of PWM (○) stock solution. The geometric means of the individual responses were normalized to percent maximal responses; the data from the three subjects were then combined and expressed as arithmetic mean ± SEM.

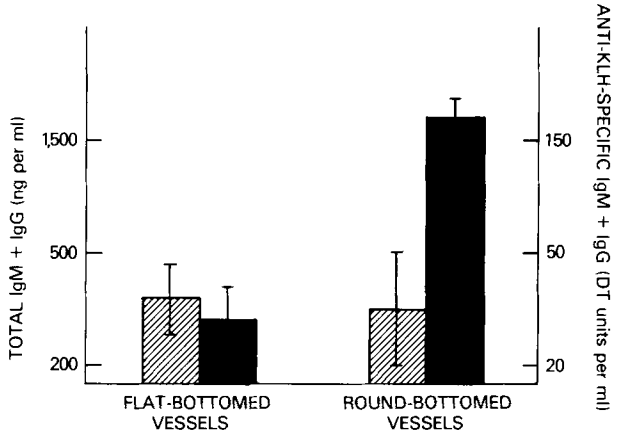

FIG. 7. Effect of culture vessel geometry on the magnitude and the specificity of antigen-induced antibody responses. Triplicate cultures were set up using cells from an immunized subject 2 wk after booster immunization in either flat-bottomed plates or round-bottomed tubes as described, and stimulated with 200 ng KLH. Specific and total IgG and IgM were measured on day 12 supernates. Data are expressed as geometric means ✕÷ SEM. ▨, specific anti-KLH antibody; ■, total antibody.

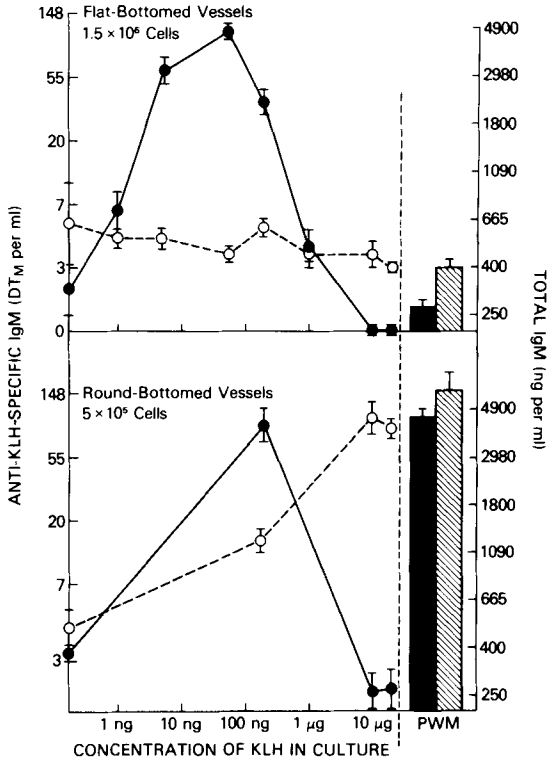

FIG. 8. Specific vs. nonspecific antigen-induced antibody responses as a function of antigen concentration, culture vessel geometry, and number of cells in culture. Triplicate cultures from the same subject were performed in both systems. Responses to PWM are given as a point of reference. Data are expressed as geometric means ✕÷ SEM. ●, ■, specific anti-KLH IgM; ○, ▧, total IgM.

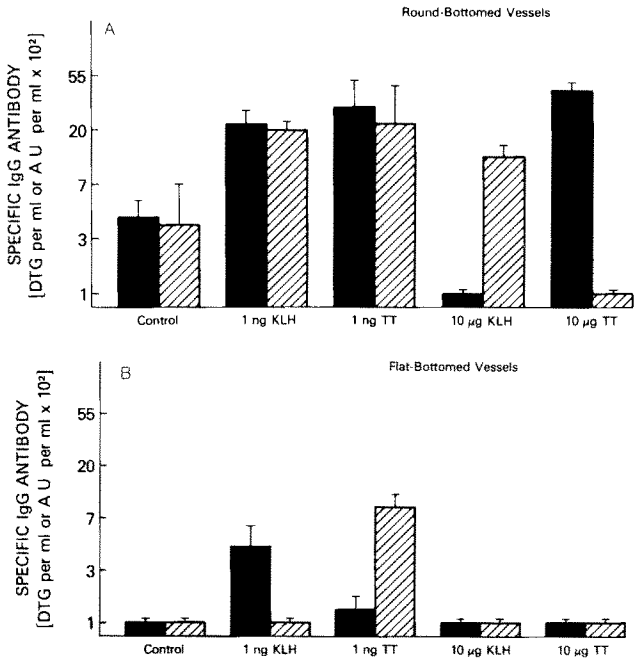

FIG. 9. Specificity of antigen-induced antibody production in a doubly immunized subject. Cultures were done in triplicate under the conditions shown. Data are expressed as geometric means ± SEM. Cultures performed in round-bottomed vessels (A) contained 5×10^5 cells, whereas those performed in flat-bottomed vessels (B) contained 1.5×10^6 cells. ■, anti-KLH antibody; ▨, anti-TT antibody.

antigen-induced antibody responses. The magnitude of the antigen-induced, antigen-specific response was the same, regardless of the culture vessel (Fig. 7). In contrast, total IgM plus total IgG production was substantially higher in the round-bottomed vessels, increasing from 350 ng/ml in the flat-bottomed vessels to 1,600 ng/ml in the round-bottomed vessels.

In another series of experiments, one set of cultures was optimized for specific antigen-induced antibody responses by culturing at 1.5×10^6 cells/ml in the flat-bottomed vessels, and another was optimized for specific and nonspecific responses by culturing at 5×10^5 cells in the round-bottomed vessels as described above. In these experiments, peak specific antibody production as well as the shapes of the dose-response curves for specific antibody production were basically the same in both systems (Fig. 8). As increasing amounts of KLH were added to the flat-bottomed cultures, total IgM produced in vitro did not vary significantly. However, total IgM production in the round-bottomed vessels showed a steady rise as additional antigen was added to culture. Maximum total antigen-induced IgM production in the round-bottomed vessels with 5×10^5 cells/culture was similar in magnitude to the amount of total IgM antibody generated by PWM and occurred at in vitro concentrations of KLH comparable with those that resulted in a peak blastogenic response (see below). Thus, flat-bottomed cultures with 1.5×10^6 cells/well yielded predominantly specific antibody responses, whereas cultures of 5×10^5 cells performed in the round-bottomed vessels resulted in both specific antibody and polyclonal Ig production.

Further evidence of the selective expression of antigen-specific responses at higher cell numbers in flat-bottomed vessels was seen in the study of individuals boosted to both KLH and TT. In cultures performed with 5×10^5 cells in round-bottomed vessels, either KLH or TT at low concentrations triggered the production of specific antibodies directed against both KLH and TT (Fig. 9 A). Higher concentrations of antigen, although they suppressed the response to the antigen in culture, continued to induce production of antibodies directed against the other, non-cross-reacting antigen to which the individual had been immunized, as part of a larger polyclonal response. Thus, the induction of antibody production by an antigen to which the subject had been recently boosted resulted in a polyclonal response, provided the cultures were carried out in round-bottomed culture vessels and was therefore nonspecific under that set of conditions. In contrast, the suppression seen in these same cultures was specific and involved only the response to the antigen in culture. Neither these nonspecific responses at low concentrations of antigen nor the polyclonal activation at high concentrations of antigen were seen in the cultures performed in the flat-bottomed vessels with 1.5×10^6 cells/well (Fig. 9 B). The responses of cultures performed in this manner were exquisitely specific for the stimulating antigen in terms of both induction and suppression of antibody responses.

Relationship of Blastogenic Responses to Antibody Production. The in vitro concentrations of KLH that gave rise to peak specific antibody production (50–200 ng/ml) were several orders of magnitude less than those that caused peak blastogenesis, and those concentrations of KLH that gave peak blastogenic responses were suppressive of specific antibody production (Fig. 10). In addition, those concentrations of antigen that did result in significant blastogenesis were the same as those that gave rise to peak nonspecific, polyclonal Ig production when cultures were performed in round-bottomed vessels with 5×10^5 cells (Fig. 8). It is of note that the peak specific antibody responses induced by antigen alone, as well as the peak blastogenic responses inducible by antigen alone were comparable in magnitude with those induced by PWM.

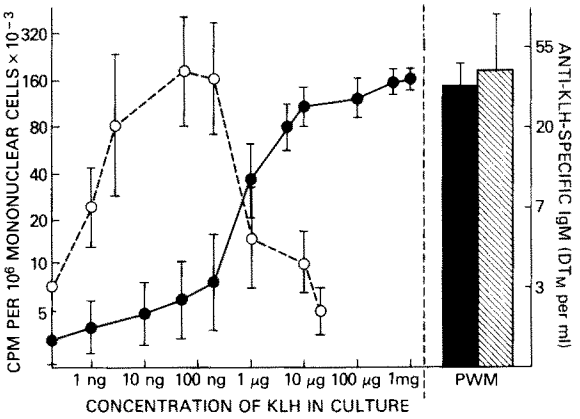

FIG. 10. Relationship of antigen-induced, antigen-specific antibody production to antigen-induced lymphocyte blast transformation. Cultures for supernate Ig production were done in round-bottomed vessels with 5×10^5 cells/1 ml culture and performed at the same time as the cultures for blast transformation. Responses to PWM are shown as a point of reference. The data from six separate individuals were pooled and expressed as geometric mean $\overset{\times}{\div}$ SEM. ●, ■, counts per minute; ○, ▨, specific anti-KLH IgM.

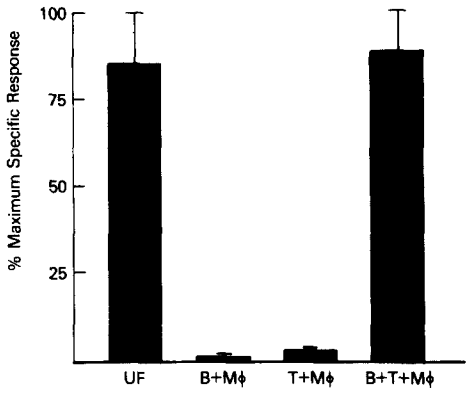

FIG. 11. T cell dependence of antigen-induced, antigen-specific antibody production. Results of triplicate cultures from two subjects were normalized to percent maximal responses and pooled. Absolute values for the individual peak responses were 49 and 123 DT_M/ml. Cultures were performed in round-bottomed vessels with 5×10^5 cells and 200 ng KLH/1 ml culture. Similar results have been obtained in flat-bottomed vessels with 1.5×10^6 cells/culture. Results are expressed as arithmetic means $\bar{X} \pm$ SEM.

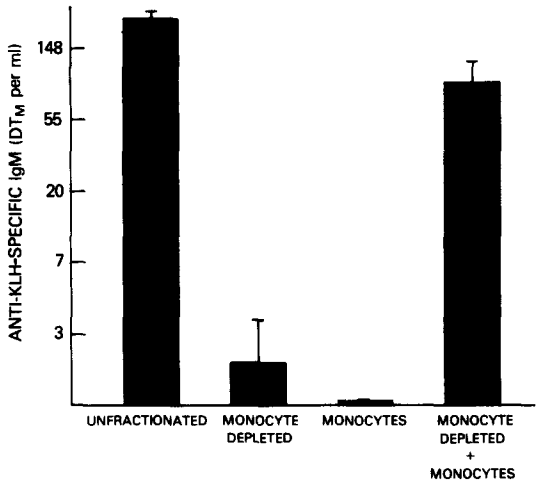

FIG. 12. Monocyte dependence of antigen-induced, antigen-specific antibody production. Cultures were performed in flat-bottomed vessels with 200 ng KLH, 7.5×10^5 monocyte-depleted cells, and/or 1.5×10^5 monocytes/1 ml culture. Results of triplicate cultures are expressed as geometric means $\bar{X} \pm$ SEM.

Cellular Requirements. The induction of in vitro, specific anti-KLH antibody production by antigen was shown to be T cell dependent (Fig. 11). 10% monocytes were added to the T cell populations to control for monocyte dependence. Neither B cells plus monocytes nor T cells plus monocytes were capable of being triggered by antigen alone to produce specific antibody. However, the combination of the two in a 1:1 ratio gave a response comparable with that of unfractionated cells.

The system was also shown to be monocyte dependent (Fig. 12). However, this phenomenon could only be demonstrated in flat-bottomed plates at low cell densities, most likely as a result of the presence of a few contaminating monocytes in the

monocyte-depleted preparations. Addition of >40% monocytes to culture decreased specific antibody production, representing either a suppressive influence and/or a diluting out of the lymphoid cells (data not shown).

Discussion

In the present study we have clearly demonstrated that in subjects that have been immunized to the soluble protein antigens KLH and/or TT, it is possible to induce with in vitro antigen alone a highly specific in vitro antibody response. The system was shown to be T cell- and monocyte-dependent, and the major variables affecting the specificity and the magnitude of the responses were antigen concentration, culture vessel geometry, and cell density.

The inability to examine easily antigen-induced, antigen-specific antibody production in man has been one of the major impediments in the study of human B cell function. In the present study, we have characterized a system that should be particularly useful in this regard. It employs soluble protein antigens that can be used for in vivo immunization and that can be haptenated for in vitro studies of carrier-hapten relationships. We have characterized the kinetics of the circulating, antigen-reactive B cell repertoire after immunization as well as the in vitro kinetics of specific antibody production, thus allowing for more predictable responses in vitro.

The induction of specific antibody production was not seen at the high concentrations of antigen that resulted in a maximal blastogenic response. However, these concentrations of antigen did result in substantial total Ig production when cultures were performed in round-bottomed vessels at lower cell number, which suggests a nonspecific induction, perhaps via recruitment or transstimulation. These findings are similar to those in the murine system, where it has been noted that T cells activated by high concentrations of antigen may provide nonspecific help (14, 15).

It is extremely important to reemphasize the fact that the use of extremely low concentrations of in vitro antigen has been the major factor in our ability to achieve high levels of specific in vitro antibody production in response to antigen alone. The relationship between blastogenesis and specific antibody production (Fig. 10) clearly demonstrates that the concentrations of antigen yielding a substantial blastogenic response (those concentrations that we and others have used for years in trying to induce antigen-specific antibody production in vitro [1, 2]), are several orders of magnitude beyond the optimal range. In fact, high concentrations of in vitro antigen, although they are able to induce large amounts of total Ig production under appropriate culture conditions (round-bottomed culture vessel, 5×10^5 cells in 1 ml), do so without the induction of antibody directed against the antigen in culture.

The critical importance of cell density and culture vessel geometry in influencing in vitro responses is apparent in this system. Conditions that favor a PWM response or a polyclonal response to antigen alone (round-bottomed vessels, low cell number) differ significantly from those favoring a highly specific response to antigen alone (flat-bottomed vessels, higher cell number). Whereas the absolute number of cells is lower in the cultures performed in the round-bottomed vessels, in fact the actual cell density is likely to be higher because of the geometric differences between the two culture vessels. The greater PWM-induced responses, as well as the polyclonal activation observed only in the round-bottomed vessels, may be explained by the need for enhanced cell to cell contact for the generation of nonspecific helper factors (16)

and the ability of such factors to bypass the need for linked recognition (17). The need for a higher absolute number of cells in the flat-bottomed cultures to obtain a maximal antigen-induced response probably reflects the frequency of the truly antigen-specific precursors at both the T and B cell levels and the need for linked recognition (18) to induce specific antibody production under these conditions. The loss of either PWM or antigen-induced specific antibody production at higher cell densities in the flat-bottomed cultures is analogous to other studies showing that help and suppression may be selected for by altering the concentration of cells (19). The fact that these changes are seen at distinctly different cell densities for PWM and antigen reinforces the concept that specific antibody production is occurring via different mechanisms for these two stimuli.

The dynamic state of the circulating cell repertoire involved in antibody production is exemplified by the changes in isotype expression seen after PWM stimulation in subjects studied 2 wk and then again 1 yr after booster immunization. The switch, with time, from an IgM to an IgG subclass of specific antibody is not observed in vivo, where IgM remains the predominant serologic anti-KLH isotype (20). Whether these changes in isotype expression are mediated at the T or B cell level remains to be determined. This same phenomenon appears to be true for in vitro antigen-induced, antigen-specific antibody production because the specific responses to a new antigen such as KLH are predominantly IgM, whereas those to TT, or to KLH 1 yr after booster immunization, are predominantly IgG.

The precise mechanism of the specific suppression seen at the high concentrations of antigen is currently under study. This suppression is refractory to low-dose irradiation of the T cells or treatment with the commercially available hybridoma antibodies directed against suppressor cells (H. C. Lane, D. J. Volkman, and A. S. Fauci, unpublished observations), suggesting that the suppression seen is not because of T cells alone and may, at least in part, be a result of the direct induction of B cell tolerance. This lack of induction of T cell suppression by high concentrations of antigen is consistent with the findings of Augustin et al. (14) and Julius and Augustin (15) that primed murine T cells induced with high concentrations of antigen do not suppress in an antibody-forming cell assay, but in fact may provide specific as well as nonspecific help.

Systems such as this, with its ability to dissect specific and nonspecific antigen-induced lymphocyte responses, should prove to be of great value in furthering our understanding of the physiology of human B cell activation and immunoregulation in normal as well as diseased states.

Summary

A highly specific and reproducible antigen-induced, antigen-specific culture and assay system for antibody production by human peripheral blood B lymphocytes has been developed. The system is clearly T cell and monocyte dependent and is independent of exogenous mitogens. The major factors in our ability to trigger specific antibody production with antigen alone have been the use of extremely low concentrations of antigen in vitro (doses several orders of magnitude below those inducing a peak blastogenic response), careful attention to in vitro cell density and culture vessel geometry, and appreciation of the kinetics of the circulating antigen-inducible B cell repertoire.

A dichotomy and overlap between antigen-induced, antigen-specific and antigen-induced, polyclonal responses was observed in the study of doubly immunized individuals. Whereas antibody responses highly specific for the antigen in culture were observed under one set of culture conditions (flat-bottomed vessels, 1.5×10^6 cells), switching to another culture system (round-bottomed vessels, 5×10^5 cells) resulted in polyclonal responses to antigen. Despite these culture condition-related differences in the induction of antibody synthesis, the suppression of specific antibody production that occurred at high concentrations of antigen was specific only for the antigen in culture.

The capability to easily and reproducibly look at truly antigen-induced, antigen-specific antibody production should be a major tool in furthering the understanding of human B cell activation and immunoregulation.

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