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Antigen-induced *in vitro* antibody production in humans: A model for B cell activation and immunoregulation

(immunoglobulin production/specific suppression/human lymphocyte/keyhole limpet hemocyanin/polyclonal triggering)

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ABSTRACT **The precise events associated with B cell activation in humans are a subject of intense investigation. It has been difficult to develop an *in vitro* model of antigen-specific triggering of antibody synthesis by human peripheral blood mononuclear cells that is independent of exogenous mitogens. In the present study a sensitive and reproducible culture system and enzyme-linked immunosorbent assay have been established wherein antigen alone is used to trigger antigen-specific antibody synthesis by mononuclear cells from subjects immunized to keyhole limpet hemocyanin (KLH). The *in vitro* antigen-induced anti-KLH response is comparable in magnitude to that induced by pokeweed mitogen, is predominantly IgM in isotype, and is accompanied by a simultaneous increase in polyclonal antibody production. Anti-KLH responses were seen at *in vitro* KLH concentrations as low as 0.05 μg/ml. However, concentrations of KLH greater than 5 μg/ml resulted in profound suppression of the anti-KLH response while continuing to trigger large amounts of total polyclonal immunoglobulin synthesis. This suppression by high concentrations of antigen was also observed in pokeweed mitogen-driven anti-KLH production. These observations are consistent with previous results from the mouse model showing a close association between antigen-specific and polyclonal responses and the phenomenon of antigen-induced, antigen-specific suppression. Thus, an *in vitro* model of antigen induction of antigen-specific antibody synthesis in human peripheral blood mononuclear cells has been demonstrated and should prove useful in exploring the mechanisms of human B cell activation and immunoregulation.**

The mechanisms of activation and immunoregulation of human B cell function have been the subject of intense interest in immunobiology over the past several years. Elucidation of the physiology of antigen-specific activation and immunoregulation of human B cells has been made difficult by the lack of simple, sensitive, and reproducible systems for the *in vitro* study of antigen-specific B cell responses. *In vitro* models of human B cell triggering have predominantly used polyclonal B cell activators (PBAs) such as pokeweed mitogen (PWM) (1, 2) with measurement of the total immunoglobulin (Ig) produced in culture by a number of diverse assay systems such as detection of intracytoplasmic Ig (3), supernatant Ig (4), and various hemolysis-in-gel plaque-forming-cell assays (5–7). Other investigators have employed the *in vivo* immunization of subjects with subsequent *in vitro* triggering of peripheral blood lymphocytes by PBAs such as PWM or lymphocyte mitogenic factor to secrete polyclonal Ig, including that with specificity against the immunizing antigen (8, 9). Human antigen-specific plaque-forming-cell assays have been reported (10–12); however, results in these systems have been inconsistent among various laboratories (1, 2). Recent studies using a radioimmunoassay have demonstrated antigen-induced triggering of specific antibody responses in peripheral blood mononuclear cells of individuals who had experienced natural infection with influenza virus (13). The present study describes a highly sensitive and reproducible system for the antigen-induced triggering and measurement of specific antibody production by peripheral blood B cells of normal subjects who had been immunized *in vivo* with the soluble antigen keyhole limpet hemocyanin (KLH). In addition, it examines the roles of antigen and PWM in the *in vitro* induction of specific and polyclonal antibody synthesis, as well as the phenomenon of antigen-specific suppression.

MATERIALS AND METHODS

Immunization. Normal human subjects received a primary subcutaneous immunization of 5 mg of KLH (Calbiochem, La Jolla, CA) followed by a 2-week booster immunization. Low levels of anti-KLH antibodies were present in the sera of all subjects prior to immunization, as has been previously reported (14). After booster immunization, a 20- to 50-fold increase of IgM and IgG anti-KLH antibodies was noted in the sera. *In vitro* studies were performed approximately 2 weeks after the booster immunization.

Cell Separation and Culture Conditions. Human peripheral blood mononuclear cells were separated by Hypaque/Ficoll gradient centrifugation and cultured in RPMI-1640 medium supplemented with gentamicin at 0.01 mg/ml, 2 mM L-glutamine, trypticase soy broth at 0.03 mg/100 ml, and 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, VA). Cells were cultured in the presence of various concentrations of KLH alone, PWM (GIBCO) alone, or a combination of PWM and KLH. PWM-stimulated cultures contained PWM at a final dilution of 1:200 of stock solution. Cultures were kept in round-bottomed 12 × 75 mm plastic tubes at a cell density of 5×10^5 mononuclear cells in a final volume of 1 ml at 37°C in 5% CO_2 in air at 100% relative humidity. Supernatants were harvested after 12 days and assayed for both anti-KLH antibody and total Ig. In certain experiments, cells were irradiated by exposure to 3600 rads (1 rad = 0.01 gray) from a ^{137}Cs source (Isomedix, Parsippany, NJ) prior to culture. In other experiments the cells were washed twice and the supernatants were replaced by antigen-free media after 1 or 4 days of incubation.

Enzyme-Linked Immunosorbent Assays (ELISA). Culture supernatants were assayed by the ELISA technique (15, 16). Anti-KLH antibody was measured on flat-bottomed microtiter plates (Immulon, Dynatech, Alexandria, VA), which were coated with 200 μl of a 1 μg/ml solution of KLH in 0.1 M sodium carbonate buffer, pH 9.6, at 4°C overnight. The plate was then

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; PBA, polyclonal B cell activator; P_i/NaCl, phosphate-buffered saline; PWM, pokeweed mitogen.

Table 1. Mean Ig production in antigen and PWM-stimulated cultures

Ig	KLH concentration in culture, μg/ml							PWM alone
	0	0.05	0.1	0.5	1.0	10	20	
Anti-KLH IgM, units/culture (±SEM)	71 (±44)	348 (±179)	357 (±187)	207 (±120)	140 (±80)	12 (±7)	6 (±5)	316 (±59)
Anti-KLH IgG, units/culture (±SEM)	4 (±4)	20 (±10)	12 (±7)	6 (±4)	5 (±3)	0	0	11 (±5)
Total IgM, ng/culture (±SEM)	7400 (±3500)	18,500 (±6,200)	21,500 (±8,900)	21,900 (±8,390)	25,800 (±10,000)	27,700 (±10,900)	31,000 (±12,300)	33,400 (±8,400)
Total IgG, ng/culture (±SEM)	440 (±320)	1,500 (±570)	1,770 (±690)	2,100 (±690)	2,200 (±840)	3,280 (±1,030)	3,220 (±1,410)	13,490 (±4,600)

The data represent the mean supernatant Ig production by mononuclear cells from six individuals immunized to KLH and cultured 2 weeks after a booster immunization.

washed three times with 0.05% Tween-20 in phosphate-buffered saline (P_i/NaCl/Tween). Samples were appropriately diluted, added to the well, and incubated 2 hr, and the plate was again washed. Anti-human IgG or IgM antibody (Fc fragment specific) conjugated to alkaline phosphatase (Orion Diagnostica, Helsinki, Finland) was then added to the plate to detect IgG or IgM anti-KLH antibody, respectively. After incubation and washing, a solution containing *p*-nitrophenylphosphate (1 mg/ml) in carbonate buffer, pH 8.6, with 0.001 M $MgCl_2$ was added to each well and subsequent development of color was detected on a multichannel spectrophotometer (Flow Laboratories). The values were related to a standard serum rich in either anti-KLH IgG or anti-KLH IgM. One unit was defined as the amount of anti-KLH IgG in a $1:10^5$ dilution of the IgG serum or the amount of anti-KLH IgM in a $1:10^4$ dilution of the IgM serum. Assays for total IgG or IgM were done in an identical manner to the anti-KLH antibody determinations except that the plates were coated with goat anti-human IgG $F(ab)_2$ or rabbit anti-human μ chain, respectively (Cappel Laboratories, Cochranville, PA). The subsequent incubation with the alkaline phosphatase-conjugated anti-human IgG or IgM (Fc fragment specific) gave the assay its isotype specificity. Monoclonal human IgM had no IgG activity and pooled human IgG had no IgM activity in the assay systems. Dilutions of supernatants were made to fall within the range of a standard curve (5–125 ng/ml) derived from dilutions of commercially titered reference sera. Values were determined in duplicate.

RESULTS

Antigen-Stimulated Ig Synthesis. The mean *in vitro* responses of peripheral blood lymphocytes from six immunized individuals to a wide range of KLH concentrations are shown in Table 1. Anti-KLH antibody responses to KLH stimulation were seen at concentrations of KLH as low as 0.05 μg/ml. Anti-KLH antibody responses increased or persisted at the higher concentrations of 0.1 and 0.5 μg/ml. However, as the concentration was increased to 1.0 μg/ml, modest suppression of the anti-KLH antibody response was seen, and at KLH concentrations of 10 and 20 μg/ml, profound suppression of the specific response occurred. Although several individuals made small amounts of IgG anti-KLH antibody, the major *in vitro* antigen-specific responses were seen in the IgM class. The anti-KLH antibody response was clearly *de novo* synthesis because irradiation (3600 rads) of the cells prior to culture resulted in abrogation of the response (17).

All concentrations of KLH that resulted in specific anti-KLH antibody responses also resulted in a marked increase in the secretion of total IgM and IgG (Table 1). Thus, KLH added to cultures of lymphocytes from these KLH-immune individuals resulted in a true polyclonal response, a proportion of which was directed against the antigen to which the individuals had been recently immunized. However, concentrations of KLH that completely suppressed specific responses continued to trigger the secretion of large amounts of total Ig (Table 1). The magnitude of the specific and total Ig responses inducible by antigen

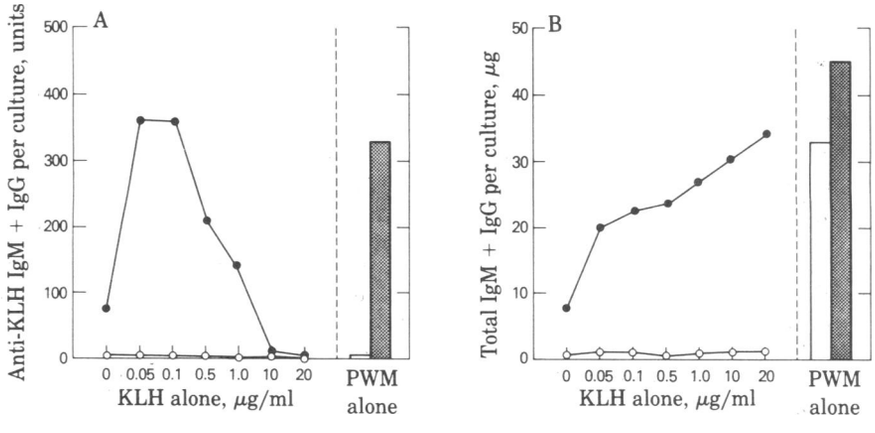

FIG. 1. Specific anti-KLH and total IgM + IgG production by lymphocytes from KLH-immunized and unimmunized individuals in response to either KLH antigen or PWM stimulation. (*A*) Anti-KLH antibody production in six KLH-immunized individuals (dark circles or bars) and in five unimmunized subjects (open circles or bars) in response to either antigen or PWM. (*B*) Corresponding total Ig production in these two groups.

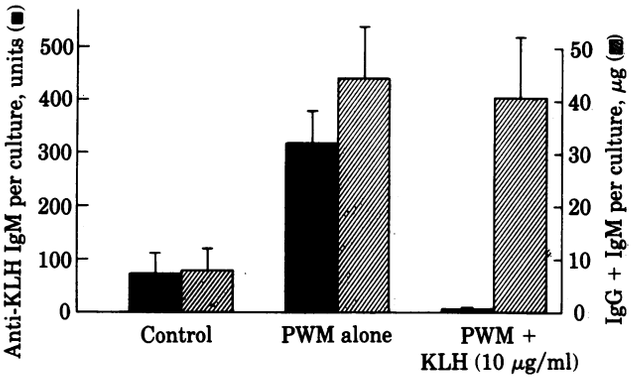

FIG. 2. Anti-KLH IgM production in PWM-stimulated cultures. Cells were cultured in media alone, in media with PWM, and in media with both PWM and KLH (10 μg/ml). The data represent the mean (±SEM) values from six individuals.

were similar to those seen in response to PWM alone (Table 1).

Five individuals who had not been immunized to KLH were studied in an identical manner and their mean responses were compared to those of the immunized group (Fig. 1). Neither KLH nor PWM was capable of triggering specific anti-KLH antibody production in the subjects who were not immunized with KLH. Although a nonspecific stimulus, PWM, produced an increased total Ig response in the unimmunized group comparable to that of the immunized subjects, stimulation with KLH failed to produce an enhanced polyclonal response in the unimmunized group.

PWM-Stimulated Ig Synthesis. The PBA PWM stimulated a relatively large amount of anti-KLH IgM synthesis and increased the total amounts of IgG and IgM production from less than 8 μg to more than 45 μg per culture (Table 1).

When KLH was added at 10 μg/ml to cultures that were stimulated with PWM, marked suppression of the anti-KLH antibody response was seen; total Ig production was not affected (Fig. 2). This observation was similar to that made in cultures stimulated with high doses of antigen alone (Table 1).

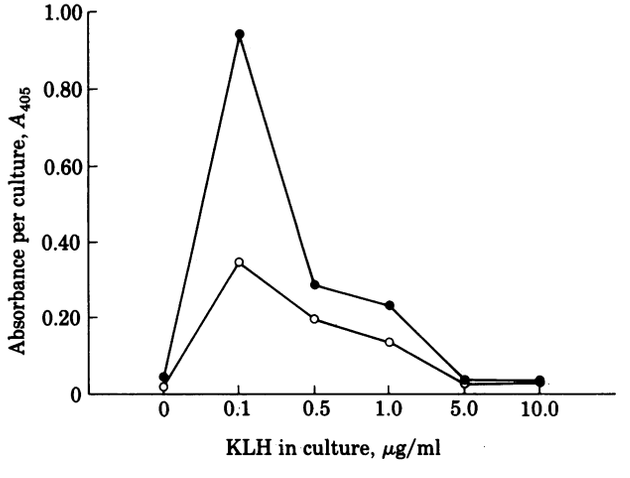

FIG. 3. Effect of KLH on the detection of anti-KLH antibody. Supernatants containing anti-KLH IgM were generated as in *Materials and Methods*. An aliquot of each supernatant was diluted 1:1 in P_i/NaCl/Tween (●). A second aliquot was diluted in P_i/NaCl/Tween plus KLH such that the final concentration of KLH in each supernatant was 20 μg/ml (○). The paired aliquots were assayed for anti-KLH IgM antibody and total Ig. The total Ig in each pair of aliquots was the same. The points represent the mean of duplicate determinations.

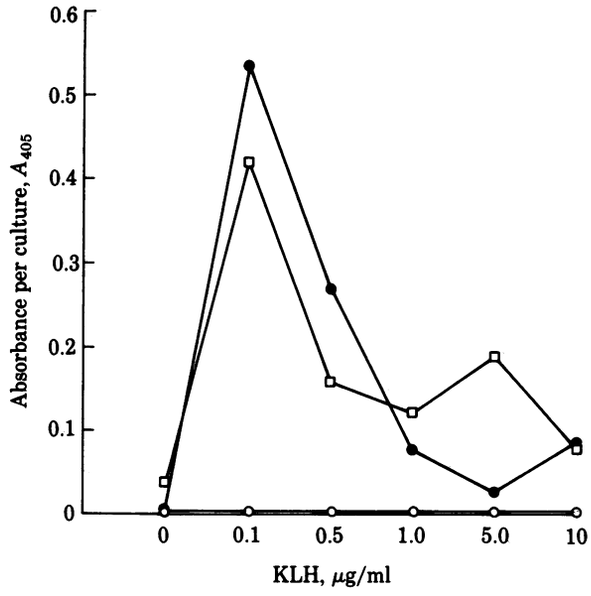

FIG. 4. Effect of the removal of antigen from culture on anti-KLH antibody production. Peripheral blood mononuclear cells were cultured at a density of 5×10^5 in 1 ml with various amounts of KLH. After either 1 (○) or 4 (●) days the antigen-containing media were removed, the cells were washed, and fresh antigen-free media were added. Some cultures were not washed (□). The supernatants were assayed for anti-KLH activity after 12 days of total incubation.

Assay Specificity. Specific suppression of the anti-KLH antibody responses in the PWM as well as the KLH-driven cultures was not due to interference by KLH in the antigen-specific ELISA. In certain experiments, anti-KLH antibody was generated in cultures in the standard manner by stimulation with low concentrations of KLH. The cultures were harvested after 12 days and amounts of KLH to yield final concentrations as high as 20 μg/ml were then added to the supernatants before anti-KLH antibody determinations were made with the ELISA. Although the ELISA absorbance was decreased in the supernatants, confirming the specificity of the assay, the anti-KLH IgM response to low concentrations of KLH was still readily apparent and the shape of the dose–response curve remained the same (Fig. 3). In addition, removal of antigen at day 4 of culture did not alter the induction of antibody synthesis by low concentrations of KLH or the suppression of specific antibody synthesis by high concentrations of KLH (Fig. 4).

DISCUSSION

The study of the mechanisms of activation and immunoregulation of human B cells has been hampered by the lack of reproducible *in vitro* systems for the triggering of human peripheral blood lymphocytes with antigen and measuring the specific antibody production (1, 2). The present study clearly demonstrates that one can reliably immunize an individual with a soluble antigen such as KLH and subsequently reproducibly trigger the *in vitro* production of specific antibody by *in vitro* stimulation with the relevant antigen alone. There are a number of interesting aspects of this system. At low concentrations of antigen in culture, the production of specific antibody is clearly inducible by antigen alone in the absence of additional PBAs. However, the specific responses induced by antigen, within the framework of the present system, are part of a polyclonal response despite the fact that true PBAs were not added to culture. Although KLH has been reported to be a PBA in certain

systems (18), this effect is weak, occurs only at extremely high concentrations of antigen, and in fact did not stimulate anti-KLH antibody production in any of our unimmunized subjects regardless of the concentration of antigen employed *in vitro*. However, in the present system, KLH at low concentrations not only can trigger antigen-specific antibody responses but also can serve as a PBA, provided the subjects have been recently immunized with KLH (Fig. 1). In this regard, it is conceivable that stimulation with any antigen that results in a polyclonal response will in fact trigger the production of antibodies of a variety of specificities, reflecting the B cell repertoire present in the circulation at the time of study. The fact that antigen-specific B cells can be triggered to antibody production by polyclonal activators provided that the relevant B cells are present in culture is demonstrated by the observation that anti-KLH antibody responses were triggered by PWM in the absence of KLH in the lymphocyte cultures of our subjects who were recently immunized with KLH (Table 1).

Regarding the dichotomy between polyclonal and specific responses, it is unclear at present whether in this *in vitro* system there exists a narrow range of KLH concentrations with which one can induce antigen-specific responses without polyclonal activation. In this regard, it has been hypothesized that polyclonal activation of one degree or another is invariably associated with every "antigen-specific" response (19). This hypothesis is in accord with *in vivo* observations, because polyclonal B cell activation has been clearly demonstrated after *in vivo* immunization with specific antigens (20).

With regard to the antigen-induced suppression observed in the present system, antigen-specific suppression in response to high antigen concentrations has been previously noted in mouse *in vitro* and *in vivo* systems and may be mediated by suppressor T cells (21, 22). The mechanisms whereby high concentrations of antigen specifically suppressed the anti-KLH response and not the total Ig response is unclear at present. This specific *in vitro* suppression provides a model for investigating the cellular interactions and requirements of this phenomenon.

Further examination of the role of antigen in inducing specific as well as polyclonal responses in systems such as this should prove useful in the delineation of the physiology of activation of human B cells and their immunoregulation.

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