INDUCTION OF POLYCLONAL ANTIBODY SYNTHESIS BY HUMAN ALLOGENEIC AND AUTOLOGOUS HELPER FACTORS*

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The role of antigen binding in B-cell activation has been investigated extensively. In several systems, it is established that both antigen binding by B cells and provision of helper activity by T cells are needed for the induction of antibody synthesis. In particular, this requirement has been evident in the studies of allogeneic effect factors in murine systems (1). In such systems, the factors generated either in vivo (2) or in vitro (3-6) were shown to help only antigen-stimulated splenic B cells.

Recent studies from this laboratory have demonstrated that in man, both allogeneic (7, 8) and autologous (9) mixed lymphocyte reactions result in helper activities. These studies have revealed some similarities but also some differences from the murine allogeneic effect model. In the present investigation, evidence has been obtained that, in the absence of specific antigens, both allogeneic and autologous helper factors result in significant polyclonal antibody synthesis. Nevertheless, these helper factors can act in concert with antigen to result in significantly higher levels of direct antigen-specific plaque-forming cells.

Materials and Methods

Preparation and Cryopreservation of Mononuclear Cells (MNC). Tonsillar tissue was obtained from routine tonsillectomy specimens. MNC from tonsillar cell suspensions and normal peripheral blood were obtained after centrifugation on Ficoll-Hypaque gradients. Tonsillar MNC were frozen in RPMI-1640, 20% fetal calf serum, and 10% dimethylsulfoxide at the rate of 1°C/min in a programmable freezer (Cryo-Med, Weld Metal Inc., Mt. Clemens, Mich.).

Preparation of Human Mixed Lymphocyte Reaction (MLR)-Derived Helper Factors

ALLOGENEIC HELPER FACTORS. 3 × 10⁶ irradiated (2,400 R) tonsillar MNC were cultured with either an equal number of normal peripheral blood MNC or 2 × 10⁶ peripheral blood T lymphocytes in 5% human serum. The serum used was from either a normal AB pool absorbed with sheep erythrocytes (SRBC) or an immunodeficient patient without isoagglutinins. After 48 h, supernates were collected, filtered through a 0.45 micron Millipore filter (Millipore Corp., Bedford, Mass.) and either used immediately or stored frozen at −70°C.

AUTOLOGOUS HELPER FACTORS. Either 3 × 10⁶ unseparated peripheral blood MNC or 2 × 10⁶ T lymphocytes were cocultured with 3 × 10⁶ irradiated (3,000 R), monocyte-depleted peripheral blood B cells for 48 h and processed as stated above for allogeneic helper factors.

Cell Cultures. 3 × 10⁶ tonsillar MNC or an equivalent number of monocyte-depleted peripheral blood MNC were cultured in Linbro tissue culture plates (16 mm: 76-033-05, Linbro

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Chemical Co., Hamden, Conn.) in 2.0 ml of sterile medium, as previously described (10). Each culture reported was tested in tandem with both a pool of normal human AB serum absorbed with SRBC and with an aliquot of serum from a patient with immunodeficiency without detectable isoagglutinins and anti-SRBC antibodies. 1 × 10⁶ washed SRBC served as specific antigens for appropriate cultures. Human helper factors were added as noted in Results.

Direct Plaque-Forming Cell (PFC) Assay, Immunofluorescent Staining for Intracytoplasmic Ig, and Measurement of DNA Synthesis. Antibody-forming cells with specificity for SRBC antigens were detected by a modification (10) of the Jerne-Nordin PFC assay. The procedure for the detection of intracellular immunoglobulin by immunofluorescence and the quantitation of DNA synthesis have been reported previously (11).

Quantitation of Immunoglobulin Secretion in Culture Supernates. In certain experiments, tonsillar MNC were cultured for 90 h, harvested, washed, and then reseeded in methionine-free medium plus 50 μCi of ³⁵S-methionine (Amersham Corp., Arlington Heights, Ill.). After 4 h, the supernates were collected. 50 μl of culture supernate were incubated for 1 h with 100 μl of Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) linked rabbit antibodies to human κ/λ-chain determinants (same antibodies used for immunofluorescence) in the presence or absence of 100 μg of purified human Fab fragments. This quantity of Fab was shown to be sufficient to saturate all of the antigen-binding activity of the immunoabsorbent. After incubation, the beads were washed six times, dried, and counted. Data presented represent the value obtained by subtracting the counts per minute of those aliquots inhibited with excess cold Fab from those assayed in the absence of Fab. The values obtained after incubation with pure Fab and precipitated with rabbit anti-Ig-Sepharose were similar to those obtained by direct precipitation with Sepharose-normal rabbit serum alone.

Results

Induction of Both Polyclonal and Antigen-Specific Antibody Production by Human Helper Factors

Allogeneic helper factors. Fig. 1 illustrates the typical effects on direct anti-SRBC PFC formation induced by allogeneic supernates when added over a broad dose range. Neither fresh nor cryopreserved tonsillar MNC alone produced PFC despite the addition of SRBC. However, after the addition of as little as 10% by volume of helper factors and SRBC, significant specific antibody secretion was detected. This rose to a maximum of >2,000 PFC/culture at a factor dose of 25%. Concentrations of allogeneic helper factors in excess of this amount resulted in a decline in the SRBC-initiated PFC response. In a series of five comparable experiments, concentrations of factor between 25 and 50% induced the highest PFC levels.

Substantial numbers of PFC also were detected in the cultures containing allogeneic helper factors to which SRBC had not been added (Fig. 1). Comparable data were obtained regardless of the serum source both for factor induction and/or its assay on PFC formation.

To confirm that human allogeneic helper factors induced polyclonal antibody production, cultured tonsillar MNC were assayed for both total immunoglobulin synthesis (plasma cells) and specific antibody secretion (anti-SRBC PFC) (Table I). Responses induced by pokeweed mitogen (PWM) are included for comparison. In all three experiments presented and in every experiment performed to date, abundant numbers of plasma cells were identified by immunofluorescent staining. Fig. 2 illustrates this dramatic induction of antibody synthesis. Similar data were obtained if anti-IgM or anti-IgG antibodies were utilized as the fluorescent probe. Furthermore, in experiments 1 and 2, high levels of anti-SRBC-specific PFC were also detected in cultures not containing SRBC. In experiment 3, few direct anti-SRBC PFC were seen
Fig. 1. Demonstration of helper activities in supernates of allogeneic cocultures of tonsillar and peripheral blood MNC. $3 \times 10^7$ irradiated (2,400 R) tonsillar MNC were cocultured for 48 h with allogeneic peripheral blood MNC in medium containing 5% AB serum. Supernates were collected, filtered, and added to cultures containing $3 \times 10^6$ cryopreserved tonsillar MNC with (X) or without (O) SRBC.

**Table I**

| Exp. No. | Cells cultured | Allogeneic helper factors | $[^3]H$-thymidine uptake (cpm) | Direct α-SRBC PFC/culture | Plasma cells/culture |
|----------|----------------|---------------------------|-------------------------------|--------------------------|---------------------|
| 1        | Tonsillar MNC alone | 0                          | 2,197                         | <5                       | <1                  |
|          | 33% Tonsillar MNC + SRBC | 5,307                     | 650                           | 15           | 20                  |
|          | 33% Tonsillar MNC + PWM  | 5,072                     | 1,050                         | 5                 | 15                  |
| 2        | Tonsillar MNC alone | 0                          | 1,279                         | <5                       | <1                  |
|          | 33% Tonsillar MNC + SRBC | 3,765                     | 350                           | 13           | 13                  |
|          | 33% Tonsillar MNC + PWM  | 1,118                     | 2,424                         | 10           | 10                  |
| 3        | Tonsillar MNC alone | 0                          | 3,594                         | <5                       | <1                  |
|          | 33% Tonsillar MNC + SRBC | 16,134                    | 20                            | 22           | 22                  |
|          | 33% Tonsillar MNC + PWM  | 3,557                     | 150                           | 25           | 25                  |

* $3 \times 10^7$ irradiated tonsillar MNC were cocultured with either $2 \times 10^7$ allogeneic peripheral blood T lymphocytes (exp. 1 + 2) or $3 \times 10^7$ allogeneic peripheral blood MNC (exp. 3) for 48 h to generate allogeneic helper factors. Factors were added to a final concentration of 33% to cultures of cryopreserved, isologous tonsillar MNC supplemented with either medium, SRBC, or PWM.

in the absence of antigen. However, secretion of immunoglobulin was documented by radioimmunoassay involving the incorporation of $^{35}$S-methionine into secreted Ig. Immunoglobulin was produced in the culture containing allogeneic helper factors without SRBC in marked excess of that produced in the control culture not containing
helper factors (3,377 vs. 100 cpm), and in amounts comparable to that secreted in the culture containing helper factors and SRBC (4,102 cpm). Thus, three separate assays documented that allogeneic helper factors resulted in polyclonal tonsillar B-cell differentiation to the antibody producing stage.

Allogeneic helper factors were also tested on monocyte-depleted peripheral blood MNC. In one such experiment, 4.8% plasma cells were detected in a culture to which the allogeneic factors were added. In the control culture without factors, only 0.1% plasma cells were observed. Similar results showing 30-50-fold increases in plasma cells were obtained with peripheral blood MNC from three different donors.

In every instance tested, the addition of SRBC to cultures of tonsillar MNC supplemented with allogeneic helper factors, resulted in a significantly enhanced level of specific PFC (Fig. 1 and Table I). This usually occurred without a major alteration in the number of plasma cells detected by immunofluorescence.

**Autologous Helper Factors.** A series of experiments similar to those reported above were performed using supernates from human autologous MLRs. In a typical experiment, these autologous helper factors were shown to induce isologous, monocyte-depleted peripheral blood MNC to synthesize Ig as determined by intracytoplasmic immunofluorescence (from 0.5-7.5% plasma cells). In addition, these factors permitted the same cells to synthesize and secrete specific antibody after SRBC were added to the cultures (≤5-115 PFC). Similar results were obtained in four experiments involving three different individuals. In some instances, allogeneic and autologous factors derived from the same donor's T cells were equally effective in differentiating tonsillar MNC to plasma cells.

**Discussion**

The preceding data demonstrate that human helper factors derived from both allogeneic and autologous mixed lymphocyte reactions, in the absence of classical antigens, induce polyclonal antibody production in unseparated tonsillar and peripheral blood MNC, as measured by the generation of plasma cells, PFC, and secreted immunoglobulin. This phenomenon has been observed in every instance studied, and
was most clearly illustrated when the number of plasma cells per culture was quantitated. When assayed in this manner, in the case of allogeneic helper factors, as many as 25% of the cultured tonsillar MNC could be identified as synthesizing Ig, a value which approximates 50% of the total B-cell number in culture. Similarly, significant numbers of direct, specific anti-SRBC PFC were induced in the majority of these cultures. Induction of PFC occurred with two different batches of human serum and with cells that had not previously been exposed to sheep erythrocytes. Similar results were obtained with autologous MLR factors. When either the allogeneic or autologous factors were assayed on peripheral blood MNC rather than tonsillar lymphocytes, the percentage of plasma cells detected per culture was less. However, after correcting for the number of B cells recovered at the end of culture, the difference in the degree of polyclonal activation was only slightly less.

This polyclonal response apparently has not been encountered in murine allogeneic effect systems (1, 3–6). In mouse spleen cultures, only antigen-initiated PFC responses were detected. The divergence of these results may be accounted for either by different origins of the lymphocytes or the conditions employed for the generation of helper factors and/or their assay.

Recently other experiments from this laboratory with leukemic cells (11) demonstrated that B-cell differentiation could be induced during an allogeneic effect. These B lymphocytes, obtained from certain patients with chronic lymphocytic leukemia, were shown to synthesize and secrete Ig after such stimulation. This differentiation obviously occurred in the absence of the antigen to which these B cells were programmed. More recent studies have shown that the addition of the allogeneic helper factors described in this paper to the leukemic cells from one of these patients resulted in similar plasma cell generation.

It must be stressed that antigen-initiated responses also were documented in the cultures described in this report. In every instance, supplementation with SRBC resulted in a higher specific PFC response.

Collectively these data suggest that the tonsil and peripheral blood contain a heterogeneous mixture of B lymphocytes which span many stages of maturation. Those cells destined to produce antibody with the aid of antigen may require a differentiative and/or proliferative step which antigen binding initiates, whereas those cells which can synthesize antibody in the absence of antigenic exposure may need only a nonspecific stimulus such as that provided by either allogeneic or autologous helper factors.

The nature of human MLR-derived helper factors is as yet undefined. Farrar reported that human allogeneic factors and SRBC induce murine spleen cells to produce specific antibody (12). Geha et al. (13) described a nonspecific mitogenic factor derived from tetanus toxoid-stimulated cells which augments B-cell production of antibody. Whether all these factors share a common stimulatory molecule remains to be determined.

Summary

Human helper factors were obtained from supernates of 48 h unidirectional allogeneic and autologous mixed lymphocyte reactions. These supernates were shown to induce the production of large amounts of immunoglobulin by tonsillar and peripheral blood mononuclear cells. Abundant polyclonal activation to antibody
production occurred in these cultures in the absence of antigenic challenge which was similar in degree to that produced by pokeweed mitogen. This was documented by quantitating plasma cells, specific plaque-forming cells, and secreted immunoglobulin. In addition, the supplementation of companion cultures with sheep erythrocytes resulted in a significant enhancement of the specific plaque-forming cell response without an appreciable change in plasma cell number or secreted Ig.

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