

TRIGGERING OF AFFINITY-ENRICHED B CELLS

Analysis of B Cell Stimulation by Antigen-specific Helper Factor or Lipopolysaccharide

I. Dissection into Proliferative and Differentiative Signals*

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Since the first demonstration by Feldmann and Basten (1) of B cell triggering in vitro by T cell-derived, antigen-specific helper factor (Hf),¹ little progress has been made in the cellular and molecular analysis of immune induction, largely because of technical difficulties. Ideally, any such analysis requires the testing of immunocompetent B cells under conditions in which the minimally required set of cooperating elements can be qualitatively and quantitatively defined. Several years ago, we reported (2-5) on the in vitro conditions for which antigen-specific, allogeneically restricted Hf triggers unprimed B cells to generate primary IgM responses to soluble serum proteins and, more recently, to histocompatibility antigens expressed on the surface of chicken erythrocytes (CRBC). We have further developed this system to the point where as few as 2,000 unprimed B cells selected for immunocompetence to a given histocompatibility haplotype by rosetting with the appropriate CRBC generate a powerful, Hf-dependent in vitro immunoglobulin M (IgM) response. Because accessory (A) cells are limiting when such small numbers of B cells are used, we were able to demonstrate (2) an absolute requirement for A cells in successful Hf-B cell interactions. More importantly, such A cells could be replaced by an A cell-derived, secretory product that is not antigen specific. In this communication, we report on the further resolution of B cell triggering into functionally distinct steps, comparing both Hf-mediated and lipopolysaccharide (LPS)-induced IgM responses. We find that both Hf and LPS, in the presence of antigen but in the absence of A cells, trigger only B cell proliferation. Hf- or LPS-induced IgM responses, however, are generated if A cells or their secreted products are added to the cultures. These A cells are radioresistant, contain cells that bear Thy-1 antigen, and secrete their product in a manner that is not antigen specific. Further experiments showed that the A cell

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¹ *Abbreviations used in this paper:* A cell, accessory cell; AFC, antibody-forming cell; B, chicken major histocompatibility locus: haplotype denoted by numeral superscript; C', complement; CRBC, chicken erythrocytes; CSF, colony stimulating factor; FCS, fetal calf serum; Hf, antigen-specific, T cell-derived helper factor; IL-2, interleukin 2; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NRFC, nonrosette-forming cells; PBS, phosphate-buffered saline; RFC, rosette-forming cells.

requirement could be completely replaced by supernatant from an activated thymoma cell line, but only for the LPS-stimulated and not for the Hf-mediated B cell response. This finding casts doubt on the generally assumed functional homology between T cell-derived and LPS-derived triggering signals. In addition, it has recently been reported (6, 7) that antigen-specific T cell help acts polyclonally on B cell blasts as opposed to small resting B cells. Contrary to this claim, we have been able to show that immunocompetent B cells, regardless of their cell cycle state, respond to Hf in a manner that is antigen specific.

Materials and Methods

Animals. CBA/J (H-2^k), C3H/HeJ (H-2^h), CBA/CAJ (H-2^k), BALB/cCr (H-2^d), and C3H/OH (H-2⁰²) female mice were bred at Ellerslie Animal Farm, University of Alberta. Mice aged 8–12 wk were used for cell cultures, and those aged 6–8 wk were used for the production of antigen-primed helper T cells. CBA/J mice were routinely used for experiments, except where noted.

Lines of chickens homozygous for the *B*² and *B*¹³ major histocompatibility haplotypes were bred by the Biosciences Animal Services, University of Alberta. These lines of chickens represent original Hyline stocks that have been described previously in detail (8).

Antigen. CRBC were kept in Alsever's solution. Before use, they were centrifuged and depleted of buffy coat, washed three times with saline, and used as antigen either in culture or in the preparation of Hf (see below).

Preparation of Antigen-primed T Cells. In vivo antigen-primed T cells were obtained according to the method of Hartmann (9). Briefly, mice were irradiated with 500 rad (¹³⁷Cs source, Gamma Cell 40 Irradiator, Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada) and injected intravenously with a mixture of 15×10^8 syngeneic thymocytes and 5×10^7 CRBC suspended in Leibowitz (L-15) medium (Gibco Canada Limited, Calgary, Alberta, Canada). On day 7, the animals were killed, and a spleen cell suspension was prepared in Ca⁺⁺- and Mg⁺⁺-free phosphate-buffered saline (PBS).

Preparation of Crude Extract from Carrier-primed T Cells. T cell-derived helper factor was obtained as previously described (4, 5), with the modification that carrier-primed T cells were suspended in Ca⁺⁺- and Mg⁺⁺-free PBS supplemented with 10⁻⁴ M EDTA. After spinning at 400 g for 7.5 min, the cells were resuspended in ice-cold phosphate buffer (5 × 10⁻² M, pH 7.4) containing 10⁻³ M EDTA at a concentration of 5 × 10⁷ cells/ml. Cells were homogenized in an ice-cooled glass-Teflon homogenizer for 20 min at a speed of 80 rpm at 6 strokes per min. Thereafter, the homogenate was spun at 35,000 g for 30 min and the supernatant used for further purification of helper factor.

Purification of T Cell-derived Hf by Sephadex G100 Chromatography. 5 ml of crude factor in diluted saline was applied to a Sephadex G-100 column (1.5 × 85 cm) that had previously been equilibrated with saline. Factor was eluted with saline at a flow rate of 17 ml/h and collected in fractions of 2.5 ml. During fractionation, absorbancy of the eluate was scanned at 280 nm. Fractions were pooled and concentrated as previously described (4, 5). The second fraction (fraction B) was concentrated by vacuum, dialyzed against saline, and passed through a Millipore filter (0.45 μ; Millipore Corp., Bedford, Mass.). The filtrate was then diluted with saline to a concentration equivalent to 500 × 10⁶ viable spleen cells/ml. This preparation was used in all experiments using Hf, unless otherwise stated.

Preparation of Antigen Affinity-purified Hf by Column Chromatography. Sepharose 4B beads (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Uppsala, Sweden) coupled (20) with poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.) were washed once in 9 × 10⁻² M phosphate buffer (pH 7.4) containing 2.2 × 10⁻¹ M sucrose. They were then mixed with thoroughly washed B₂ CRBC in the same buffer at a ratio of 2 ml beads/0.5 ml of CRBC (packed volume). The mixture was allowed to stand for 30 min at room temperature, after which glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.) was added dropwise to a final concentration of 0.25%. During fixation with glutaraldehyde, the mixture was gently stirred. After fixation at 37° for 90 min, the CRBC-coupled beads were washed three times in cold PBS and mixed with glycylgly-

cine (60 mg/100 ml of PBS), allowed to stand for 30 min at room temperature, washed in PBS, and used to set up a column (0.9 × 3 cm). The column was washed with 2 M NaCl and buffered with PBS. A preparation of crude extract from B^2 -primed T cells was applied to the column at a flow rate of ~6 ml/h. After washing with PBS (five column volumes), 2 M NaCl (three column volumes) was added for elution of bound material. Both the bound material that had been eluted and unbound (pass-through) material were concentrated by vacuum filtration, dialyzed extensively against PBS (Dulbecco), and passed through a Millipore filter (0.45 μ ; Millipore Corp.). The filtrate was then diluted with PBS to a concentration equivalent to 500×10^6 viable spleen cells/ml.

Preparation of Rosette-forming Cells (RFC) and Nonrosette-forming Cells (NRFC). RFC were prepared according to a modification of the method of McConnell et al. (10). Briefly, unprimed mouse spleen cells in Dulbecco's PBS supplemented with 5% fetal calf serum (FCS; Gibco Canada Limited, Calgary, Alberta, Canada) were fractionated by means of an Isopaque-Ficoll (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) gradient spun at 2,000 g for 30 min at 20°C. After washing, a suspension of 10^8 cells/ml was taken from the middle layer of the gradient and mixed with 5×10^7 CRBC/ml of cell suspension and kept at 4°C for a minimum of 60 min. The mixture was then separated on an Isopaque-Ficoll gradient, as above. Using this method, the pellet yielded, along with CRBC, $\sim 2 \times 10^5$ to 4×10^5 RFC per 10^8 middle layer spleen cells. Over 90% of these RFC were surface immunoglobulin positive, and anti-Thy-1.2 and C' treatment of spleen does not affect the yield of RFC (unpublished observations). The pellet was washed three times before use as a source of RFC with PBS containing 5% FCS. The remaining nonrosetted cells were irradiated with 1,500 rad and were washed three times in PBS containing 5% FCS before use as a source of A cells at a concentration of 2×10^6 cells per culture.

Tissue Culture. RFC were cultured in triplicate at a concentration of 2×10^3 RFC in 0.2 ml of Click's culture medium (11) containing 10% Rehatuin FCS (Reheis Chemical Co., Chicago, Ill.) in Linbro microculture plates (Flow Laboratories, Rockville, Md.) and placed in an incubator gassed with 10% CO₂ in air. 2-mercaptoethanol at a final concentration of 5×10^{-5} M was added to all cultures.

Adherent Cells. A suspension of 8×10^7 spleen cells in 8 ml of complete Click's medium was incubated at 37°C in 100×20 -mm tissue culture dishes (Corning Glass Works, Science Products Div., Corning, N. Y.). After 1 1/2 h, the adherent cells were gently resuspended, transferred to new dishes, and incubated for an additional 1 h. The same process was repeated once more, and adherent cells were harvested using a rubber policeman, then irradiated with 1,500 rad and washed before use in culture.

Adherent Cell Supernatant. Irradiated adherent spleen cells were cultured for 3 d at a concentration of 2×10^6 cells/ml in 10 ml of Click's medium in 100×20 -mm tissue culture dishes. Cultures were maintained in a 10% CO₂ in an air atmosphere at 37°C. Culture supernatants were collected and passed through 0.45- μ Millipore filters. The concentration of supernatant is expressed in terms of volume-equivalents per 10^6 viable cells from which it was obtained.

Anti-Thy-1.2 Treatment. Monoclonal anti-Thy-1.2 antibody was obtained from New England Nuclear, Lachine, Quebec. The method used was that of Shaw et al. (12, 13). Briefly, spleen cells were suspended at 2×10^7 cells/ml in antibody reconstituted with medium or in medium alone (Leibovitz + 0.1% gelatin) and incubated at 37° for 20 min, after which the cells were pelleted and resuspended in the original volume, using absorbed rabbit complement (C') diluted 1/5 in medium. After 45-min incubation at 37°, cells were pelleted, resuspended in culture medium, and the viability after treatment was determined by eosin dye exclusion. Treated cells were always used in numbers equivalent to the untreated control to avoid selective enrichment of the remaining cells.

IL-2. Partially purified supernatant from an IL-2-secreting T lymphoma line (EL4) was kindly provided by Dr. V. Paetkau and B. Caplan, Department of Biochemistry, University of Alberta. This cell line, obtained from Dr. J. Farrar (National Institutes of Health, Bethesda, Md.) has been described in detail (14). The amount of IL-2 in the supernatant was quantitated in the T cell growth factor assay of Gillis (15).

LPS. LPS from *E. coli* serotype 055:B5 (Sigma Chemical Co.) was used at a concentration

of 10 $\mu\text{g}/\text{ml}$ of culture medium. The concentration used for the generation of B cell blasts was 10 $\mu\text{g}/\text{ml}$.

Antibody-forming Cells (AFC). The number of AFC was determined by the direct hemolytic plaque assay of Cunningham (16). CRBC of the appropriate haplotype were used as targets at a final concentration of 1.5×10^8 cells/ml in Mishell-Dutton's balanced salt solution (17). Immune responses were expressed in terms of AFC/culture.

Cell Proliferation. The degree of cell proliferation was measured in a Beckman scintillation counter (Beckman Instruments, Inc. Fullerton Calif.) after an 18-h pulse with 5 $\mu\text{Ci}/\text{ml}$ of [^3H]thymidine (2 Ci/mmol) (Amersham Radiochemicals, Oakville, Canada). Results were expressed as counts per minute per culture and measurements carried out on day 4 of culture, unless stated otherwise.

Antibodies. Monoclonal anti-I-A^k (K, γ 2b) (116-32) antibody was kindly provided by Dr. G. Hämmerling, Department of Genetics, University of Cologne. The serological properties of this antibody have been described (18). Anti-H-2^d antibody was kindly provided by Dr. T. Wegmann from this department. The serological properties of this antibody have been previously described (19). Rabbit anti-mouse IgG antibody was purchased from N. L. Cappel Laboratories, Inc., Cochranville, Pa. Antibodies were coupled (20) to Sephadex 4B beads, and putative Hf was absorbed by the batch method.

Results

Results of the IgM Response into Proliferative and Differentiative Events. We demonstrated that the initiation of a specific *in vitro* IgM response by unprimed murine B cells requires three components—antigen, antigen-specific T helper cells, and A cells, in the form of irradiated NRFC or adherent cells—and that the requirement for T helper cells and A cells can be replaced by products extracted from or secreted by these two cell classes, respectively (2). In view of the conflicting reports regarding the nature of signals provided to B cells by antigen (21-23), helper T cells (22, 24), and A cells (7, 25, 26), we performed reconstitution experiments in which B cell proliferation and differentiation were measured in response to antigen and T cell-derived Hf, with or without A cell culture supernatant. 2×10^3 unprimed spleen cells, specifically enriched by rosetting with CRBC of the B^2 MHC haplotype, were cultured with B^2 CRBC alone (B^2 RFC) or with the addition of B^2 -specific Hf or A cell culture supernatant, or with both. Results show that immunocompetent B cells neither proliferate nor differentiate into AFC in the presence of antigen alone (Table I). However, in the presence of Hf and antigen but in the absence of A cell culture supernatant, a proliferative response is observed; in spite of this proliferative response, few AFC are generated. Neither cell division nor differentiation is observed in the presence of antigen and A cell supernatant. Full reconstitution of the AFC response requires signals derived from both antigen-specific Hf and A cell culture supernatants. This AFC response is specific for B^2 RFC but shows a low degree of cross-reactivity with B^{13} RFC, as previously discussed (2).

Parallel experiments using LPS as a nonspecific activator rather than Hf were also performed. In the presence of LPS but in the absence of A cell culture supernatant, B cells enriched for responsiveness to the B^2 CRBC antigen undergo marked proliferation. Despite intense cell division, no significant degree of B cell differentiation into AFC is observed unless cultures are provided with both LPS and A cell culture supernatant. This AFC response, unlike that seen with Hf, is not specific for B^2 RFC.

We examined the specificity of the proliferative response using Hf purified by antigen-affinity column chromatography. B^2 or B^{13} RFC were cultured alone or with various concentrations of affinity-bound or unbound Hf B^2 (Fig. 1). Results show

TABLE I
Separation of the IgM Response into Proliferative and Differentiative Events

RFC	Hf or LPS	Adherent cell supernatant	[³ H]thymidine uptake (cpm) $\bar{x} \pm$ SEM	AFC/culture* $\bar{x} \pm$ SEM
<i>B</i> ²	—	—	317 \pm 82‡	<1
<i>B</i> ²	Hf <i>B</i> ²	—	12,307 \pm 825	43 \pm 13
<i>B</i> ²	—	+	282 \pm 64	<1
<i>B</i> ²	Hf <i>B</i> ²	+	10,072 \pm 832	408 \pm 124
<i>B</i> ¹³	Hf <i>B</i> ²	+	ND§	72 \pm 20
<i>B</i> ²	—	—	415 \pm 35	<1
<i>B</i> ²	LPS	—	63,115 \pm 1,831	44 \pm 18
<i>B</i> ²	—	+	440 \pm 35	<1
<i>B</i> ²	LPS	+	59,049 \pm 669	432 \pm 30
<i>B</i> ¹³	LPS	+	ND	398 \pm 45

2×10^3 RFC were cultured in the presence or absence of 5×10^6 cell equivalents Hf, or 10 μ g/ml LPS, and/or 2×10^4 cell equivalents of adherent cell supernatant for 4 d. [³H]thymidine uptake and numbers of AFC were counted on day 4.

Note: Hf or LPS, in the absence of A cell supernatant, induce RFC proliferation; the addition of A cell supernatant promotes differentiation into AFC. *B*² Hf-induced differentiation is specific for *B*² RFC, whereas LPS-induced differentiation is not.

* AFC produced by *B*² RFC were assayed on *B*² target CRBC; those produced by *B*¹³ RFC were assayed on *B*¹³ CRBC.

‡ Control [³H]thymidine uptake by 2×10^3 Thy-1.2 and A-cell depleted spleen cells equals 331 ± 14 .

§ Not done.

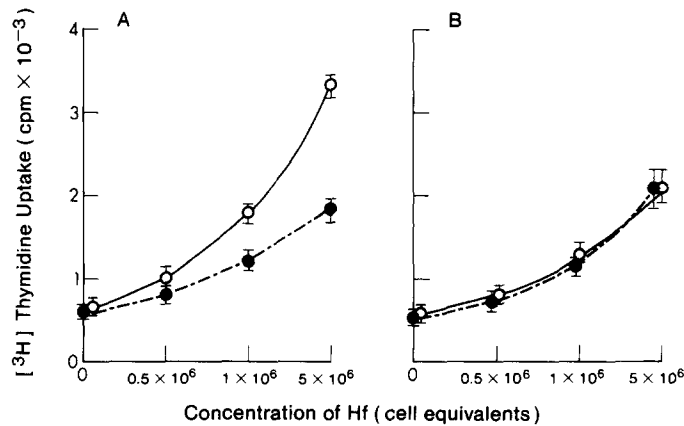


FIG. 1. Proliferative activity of affinity-purified Hf. 2×10^3 *B*² (○) or *B*¹³ (●) RFC were cultured for 4 d with various concentrations of *B*² affinity-column purified Hf (expressed in equivalents of 1×10^6 helper T cells from which it was extracted). Results are expressed as means \pm SEM of [³H]thymidine uptake on day 4 of culture. (A), bound Hf *B*²; (B), unbound Hf *B*².

that the proliferative activity of affinity-bound Hf is antigen specific. Although there is some stimulation of *B*¹³ RFC with both bound and unbound Hf *B*², an additional *B*²-specific proliferative component can clearly be demonstrated using affinity-bound Hf *B*² with *B*² RFC.

Resting B Cells and B Cell Blasts Are Triggered Antigen-specifically by Hf. Antigen-specific T cell help for resting B cells has been reported to polyclonally stimulate B cell blasts (6, 7). We examined this observation using either LPS-treated or untreated B cells, both of which were enriched for responsiveness to *B*² CRBC (Table II).

TABLE II
Unprimed and 48-h LPS-primed B Cells Depend on Antigen-specific Hf for Immune Triggering

Contents of culture			AFC/culture $\bar{x} \pm \text{SEM}$	
RFC	Hf	NRFC	Unprimed cells	LPS primed blasts
B^{13}	B^{13}	B^{13}	300 ± 54	296 ± 14
B^{13}	B^2	B^{13}	86 ± 12	62 ± 13
B^2	B^2	B^2	373 ± 20	292 ± 20
B^2	B^{13}	B^2	80 ± 37	36 ± 15

Unprimed B cells; 2×10^3 RFC were cultured with 2×10^6 irradiated NRFC for 4 d in the presence of 5×10^6 cell equivalents of B^{13} - or B^2 -specific Hf. LPS-primed B cells; 2×10^3 RFC were cultured with $10 \mu\text{g/ml}$ of LPS for 48 h, washed, and recultured for an additional 48 h with 5×10^6 cell equivalents of B^{13} - or B^2 -specific Hf. Both groups were assayed on day 4 of culture.

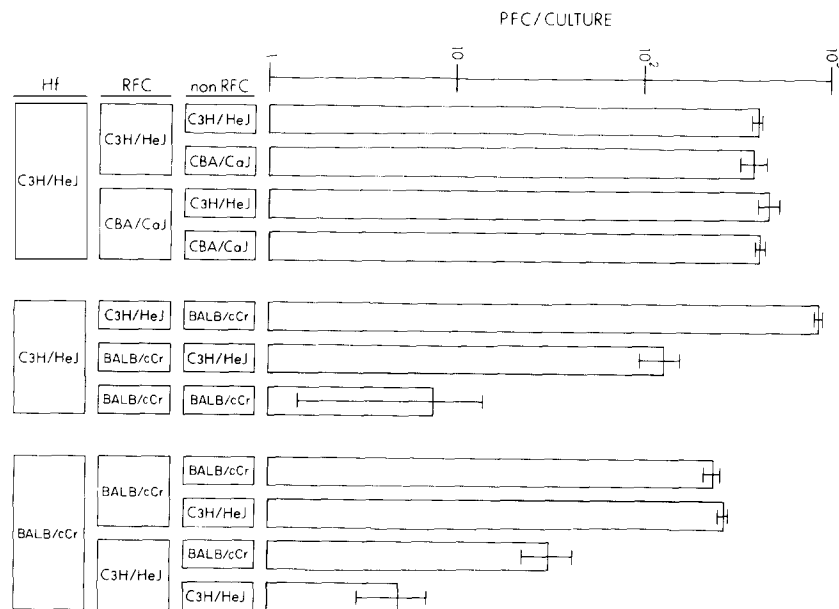


FIG. 2. Allogeneic restriction by H-2 controls Hf-B cell cooperation and is enhanced by A cells. Note that non-H-2 strain differences have no influence on Hf-B cell cooperation.

Results show that both LPS-induced B cell blasts as well as resting B cells are optimally triggered to produce AFC only in the presence of Hf of appropriate antigen specificity. We conclude that activated B cell blasts are no different from unstimulated B cells in their response to antigen-specific Hf.

Major Histocompatibility Complex (MHC) Control of the IgM Response Involves Hf, B Cells, and A Cells. In an earlier publication (3), we demonstrated the existence of MHC restriction at the level of Hf-B cell cooperation. In view of previous and current studies on the definition of a minimal set of cooperating elements in B cell triggering, we felt it important to determine the extent to which A cells are involved in mechanisms of allogeneically restricted Hf-B cell interaction. Accordingly, reconstitution experiments were set up, consisting of different syngeneic and allogeneic combinations of antigen-specific Hf, B cells, and A cells together with the appropriate antigen. We found that

TABLE III
Hf Is Absorbed by Anti-IA^k, but Not by Anti-H-2^d or Anti-IgG Antiserum

Hf absorbed by	AFC/culture (\pm SEM)*
Sepharose 4B	248 \pm 20
Anti-IA ^k -Sepharose 4B	12 \pm 18
Anti-H-2 ^d -Sepharose 4B	156 \pm 32
Anti-IgG-Sepharose 4B	260 \pm 25

2×10^3 RFC and 2×10^6 NRFC from CBA/J mice were cultured for 4 d in the presence of 5×10^6 cell equivalents of Hf B² that had previously been absorbed on sepharose alone, or sepharose coupled to anti-IA^k, anti-H-2^d, or anti-IgG antibody.

* Assayed on B² CRBC.

full immunocompetence is observed only when Hf and B cells are H-2 compatible (Fig. 2), in which case cooperation between Hf and B cells in the presence of either syngeneic or allogeneic A cells is equally efficient. However, although an allogeneic combination of Hf and B cells reduces responsiveness by $\sim 70\%$, incompatibility of Hf with both B cells and A cells entirely abolishes the AFC response. This finding agrees in principle with the original observation of allogeneic restriction between cooperating T and B cells (27) and furthermore designates the A cell as part of its underlying mechanism.

In view of this and the data described above, we decided to look for the presence of I-A subregion-encoded determinants on Hf (Table III). As indicated by affinity chromatography using monoclonal anti-I-A^k antibody, Hf contains these determinants, whereas conventionally raised anti-H-2^d and anti-IgG antibody fail to bind Hf.

From these results, we conclude that allogeneic restriction of the IgM response in vitro involves Hf and B cells as well as A cells. The role of I-A-encoded products present on our Hf in this collaborative response remains undefined.

Anti-Thy-1.2 Treatment Abrogates A Cell Function in the Hf or LPS-mediated AFC Response. There has been conflicting evidence concerning the requirement for T cells as accessory cells, both for Hf-mediated (24, 28) and for LPS-induced (29-32) antibody responses.

In previous experiments, we reported (2) that spleen cells treated with conventional anti-Thy-1.2 anti-serum and C' were as responsive to Hf-mediated immune triggering as normal spleen cells. In our search for the identity of the A cell capable of reconstituting the AFC response, as previously described (2), we have reexamined these earlier data, using experimental conditions in which T cells are more likely to be present in limiting numbers and using monoclonal high titer anti-Thy-1.2 antibody for the depletion of participating cell populations.

Under these more stringent conditions, differentiation after Hf- or LPS-induced B cell proliferation depends on the presence of a radioresistant, Thy-1.2-positive cell found in the NRFC population. Thus, anti-Thy-1.2 and C' treatment of A cells used to reconstitute Hf- or LPS-stimulated B cell cultures resulted in a 80-90% reduction in numbers of AFC compared to those of control cultures (Table IV). Anti-Thy-1.2 and C' treatment of RFC did not affect their immunocompetence because such treated cells respond well in the presence of A cells treated with C' only.

These results indicate that Thy-1.2-bearing cells must be present in the A cell population to facilitate Hf- or LPS-induced antibody production.

TABLE IV
Anti-Thy-1.2 Treatment Abrogates A Cell Participation in the Hf- or LPS-mediated IgM Response

Contents of culture		AFC/culture ($\bar{x} \pm \text{SEM}$)	
RFC	NRFC	Hf B^{13}	LPS
C'	C'	540 \pm 129	1,244 \pm 84
α Thy-1.2 + C'	C'	497 \pm 12	1,080 \pm 108
C'	α Thy-1.2 + C'	72 \pm 11	160 \pm 68
α Thy-1.2 + C'	α Thy-1.2 + C'	58 \pm 27	75 \pm 32

Unprimed spleen cells were treated with C' or anti-Thy-1.2 plus C', incubated with B^{13} CRBC, then fractionated into RFC and NRFC. RFC yield was unaffected by anti-Thy-1.2 plus C' treatment. 2×10^3 RFC were cultured with 2×10^6 C' treated NRFC or the equivalent number of cells remaining after anti-Thy-1.2 plus C' treatment, and 5×10^6 cell equivalents of Hf or 10 $\mu\text{g/ml}$ of LPS. AFC were measured on day 4.

TABLE V
Supernatant from an Activated Thymoma Cell Line (EL4) Replaces the Requirement for Accessory Cells in the LPS-induced but Not in the Hf-mediated IgM Response

RFC	Concentration of IL-2 on EL4 supernatant	AFC/culture ($\bar{x} \pm \text{SEM}$)		
		No Hf or LPS	Hf B^{13}	LPS
B^{13}	—	<1	<1	<1
B^{13}	1:20,000	<1	<1	<1
B^{13}	1:6,000	<1	<1	50 \pm 27
B^{13}	1:2,000	<1	<1	84 \pm 36
B^{13}	1:600	<1	<1	224 \pm 34
B^{13}	1:200	<1	<1	228 \pm 16
B^{13}	1:60	<1	<1	396 \pm 9
B^{13} anti-Thy-1.2 + C'	1:60	<1	<1	360 \pm 51*
B^{13} C'	1:60	<1	<1	340 \pm 76*
B^{13}	NRFC	14 \pm 5‡	296 \pm 64‡	356 \pm 27‡

2×10^3 RFC were cultured for 4 d in the presence or absence of 5×10^6 cell equivalents of Hf or of 10 $\mu\text{g/ml}$ LPS, and with varying concentrations of purified supernatant from an IL-2 producing cell line. Concentrations of IL-2 in the supernatant are expressed in terms of activity in a standard thymocyte proliferation assay for IL-2, where a 1:200 dilution gives maximum thymocyte proliferation.

* Spleen cells were preincubated with C' or with anti-Thy-1.2 antibody and C', washed, incubated with B^{13} CRBC, and fractionated into RFC and NRFC.

‡ Control cultures containing 2×10^3 RFC and 2×10^6 NRFC alone or with Hf or LPS.

Supernatant from an Activated, IL-2-secreting T Lymphoma Cell Line Replaces A Cells in the LPS-induced but Not the Hf-mediated IgM Response. Having demonstrated a requirement for Thy-1.2-bearing, radioresistant and nonantigen-specific A cells in the LPS- and Hf-induced IgM response, we explored the possibility that the culture supernatant of such cells might be identical with IL-2. This T cell-derived lymphokine has been shown (33, 34) to support an antibody response to heterologous erythrocyte antigens by spleen cells from *nu/nu* mice. In our experiments, partially purified culture supernatant from an IL-2-secreting T lymphoma (EL4) was substituted for A cells. EL4 supernatant in the absence of Hf or LPS fails to stimulate AFC production (Table V). However, in the presence of LPS, the supernatant reconstitutes the AFC response in a dose-dependent fashion; at appropriate concentrations, the response is comparable in magnitude with that of A cell (NRFC)-reconstituted control cultures.

The optimal concentration of supernatant required for reconstitution of the LPS response is comparable to that needed for maximum proliferation of thymocytes in a standard assay for IL-2 (15). Therefore, to investigate the possibility that EL4 supernatant might be acting via a small number of contaminating T cells in the RFC population, the latter were depleted of putative Thy-1.2-bearing cells before culture. The results show that the capacity of EL4 supernatant to generate AFC from LPS-stimulated B cells is independent of Thy-1.2-positive cells. Supernatant from which colony stimulating factor (CSF), which is coproduced by the cell line, had been removed by ion-exchange and phenyl-sepharose chromatography did not affect its reconstituting potency for AFC production (data not shown).

In marked contrast to its effect on LPS-stimulated B cell cultures, the EL4 supernatant, when tested over a wide range of concentrations (including those that were effective in triggering the LPS-mediated response), failed to promote an Hf-induced AFC response. Control cultures using NRFC instead of supernatant were readily reconstituted, indicating the functional integrity of the Hf used in the above experiment. We conclude that the triggering of B cells into AFC by antigen-specific Hf requires A cells that are either different from or supplementary to those needed for AFC responses induced by the polyclonal activator LPS.

Discussion

The purpose of this study was to arrive at a definition of the cellular and molecular requirements for Hf-mediated and LPS-induced (for purposes of comparison) B cell proliferation and differentiation into AFC.

This has been made possible by culturing B cells specific for a given antigen in numbers small enough to limit the participation of accessory cells and to reduce the background activity to an average of <1 AFC per culture. The stepwise reconstitution of such B cell cultures with other cooperative elements in either soluble or cellular form has permitted the dissection of the IgM response by B lymphocytes into a proliferative and differentiative event, each of these depending on a distinct triggering signal.

Of considerable interest in this respect is whether or not the immunocompetent cell's triggering mechanism is measurably affected by antigen binding alone in the absence of Hf and A cells. B cell proliferative responses induced by antigen alone have been described in several reports (21, 23). In agreement with others (22), however, we found no evidence that antigen alone induces B cells to undergo proliferation. Our data show that RFC in the presence of antigen and Hf but in the absence of A cells undergo proliferation with little differentiation into AFC. Using Hf purified by affinity chromatography, both an antigen-specific component and a nonantigen-specific component could be demonstrated for this proliferation; B^2 -specific Hf was shown to induce a proliferative response with B^{13} -specific RFC, which was of significantly lower magnitude than with B^2 -specific RFC. This is in the same range as the functional specificity of our Hf with regard to antibody formation, when the Hf is reconstituted with A cell products (2). It is possible that the unbound material inducing nonspecific proliferation might be due to a contaminant of the Hf preparation superimposing its effect on an antigen-specific, Hf-dependent proliferative signal. Such an explanation includes the possibility that nonspecifically induced proliferation might be due to the presence of biologically active subunits derived from

the dissociation of an antigen-specific molecular complex whose stability *in vitro* is short-lived.

We previously showed (2) that the A cells required to reconstitute an Hf-dependent IgM response are adherent, radioresistant, and not antigen specific in their activity. We now extend these findings to demonstrate that A cells secrete a product(s) that, in the presence of Hf and Ag, can trigger the differentiation of immunocompetent B cells into AFC. In the absence of Hf, however, the A cells or their products have no effect on B cells. This is compatible with the suggestion that B cells might become receptive to nonspecific differentiative signals at some point during the course of an antigen-specific response (25, 35).

In our previous work (2), using conventionally prepared anti-theta serum rather than monoclonal Thy-1.2 antibody, this treatment of spleen cells did not affect their response to Hf. In this study, using monoclonal antibody and an irradiated population of A cells, we demonstrate that the latter must contain Thy-1.2-bearing cells. These new findings suggest that at least one of the classes responsible for the reconstituting effect of the A cell population might bear a low density of Thy-1.2 antigen (thus, being relatively insensitive to killing by conventional serological means) or that these cells can exert their biologic effect even when present in very low numbers. These data are also compatible in principle with reports by other investigators (24, 35-39) who have described the collaboration between different classes of helper T cells that are readily distinguishable by a number of criteria. In one such model, one class of T cells releases an antigen-specific signal that leads to B cell proliferation, whereas a second type of T cell releases nonantigen-specific factors that promote B cell differentiation (35). Another group of investigators (37, 38) has described cooperation between two distinct classes of T cells in the secondary humoral response; one of the participating T cell classes is an adherent cell whose mode of action appears to be polyclonal.

The question of how Thy-1.2-negative A cells are contributing to B cell differentiation is currently under investigation. The requirement for macrophages in Hf-mediated responses has been reported (40). It is, in this context, worth remembering that T cells or their products have been shown to activate macrophages (41), and it is possible that our data reflect the necessity for a T cell to provide such a macrophage-activating factor. Experiments using cultured bone marrow-derived macrophages, whose cooperative potential requires a mitogen-activated T cell, as a source of A cells are currently underway.

For comparison, we also analyzed the *in vitro* immune response to rosette-forming B cells, using the polyclonal activator LPS, again under conditions in which A cells are limiting. As with Hf, LPS induced only B cell proliferation in these cultures. It is difficult to reconcile this result with reports that LPS by itself induces B cells to differentiate into AFC. Our LPS-stimulated cultures had to be reconstituted with A cells or their culture supernatant to observe direct plaque-forming cells; it is important to note that no differentiation into AFC was observed with A cells or A cell supernatant alone in the absence of LPS. The finding that depletion of Thy-1.2-bearing cells results in the ablation of the LPS-dependent IgM response is in contrast to the reported T independence of this response (31, 32). However, our findings agree with those of other investigators (29, 30), who have shown that under appropriate conditions it is possible to demonstrate the T cell dependence of the LPS-induced

response. It is probable, therefore, that these cells have also become limiting in our RFC population under the culture condition we used, thereby unmasking the T cell dependence of this response.

The parallels between the proliferative response induced by both Hf and LPS and the requirement in both cases for A cells (including Thy-1.2-bearing cells) to reconstitute the IgM response might initially suggest that they deliver signals to B cells via similar pathways, rendering them receptive to the same differentiative signal derived from A cells or their supernatant. This is most likely incorrect, however, as demonstrated by our attempt to replace A cells with purified supernatant derived from an IL-2-secreting T lymphoma cell line (EL4). The reconstitution from a merely proliferative to a fully differentiated IgM response occurred only in the presence of LPS but not of Hf. This strongly suggests that the Hf-mediated response has either different or additional A cell requirements and indicates that IL-2, at least by itself, cannot provide the A cell signal for Hf-primed B cells.

Among its spectrum of activities, IL-2 has been described to restore the anti-SRBC antibody response by spleen cells of nude mice (33, 34) and to act synergistically with macrophages in promoting B cell differentiation (25). In our system, also, the magnitude of the IgM response was directly proportional to the concentration of IL-2 in the EL4 supernatant. We note, however, that by itself, EL4 supernatant could not induce the differentiation of B cells into IgM-secreting cells; its activity required the presence of LPS, which induced B cell proliferation. As discussed by Delovitch (42), IL-2, like the TRF of Schimpl and Wecker (21) might belong to a class of lymphokines that can enhance antibody secretion by previously activated B cells. In addition, preliminary experiments show that the activity of the EL4 supernatant is absorbed out by activated T cells, which is one of the properties of IL-2 (43).

Although the above data on the LPS-induced response agree with the notion that the reconstitution of the A cell signal could be mediated by IL-2, we are nevertheless exploring the possibility that our findings might be due to some other lymphokine concomitantly secreted by the tumor, such as the TRF described by Schimpl and Wecker (21). In this regard, we point out that the differentiation of LPS blasts into IgM-secreting cells in the presence of EL4 supernatant was not affected by anti-Thy-1.2 and C' treatment of the responder RFC population. This suggests that the active component in the supernatant could be acting directly on the B cells rather than indirectly inducing T cells to generate a B cell differentiation factor.

It has recently been proposed (6, 7) that antigen-specific T cell help, while acting specifically on resting B cells, stimulates B cell blasts polyclonally, regardless of their antigen specificity. Using our antigen-specific, T cell-derived Hf, we cannot confirm this finding. In our system, the generation of the IgM response by B cell blasts still depends on the presence of Hf of the appropriate antigen specificity, as is the case with cultures containing resting B cells. This indicates that transformation of a B cell into the blast state is not sufficient to make it susceptible to nonantigen-specific triggering by Hf, and we conclude that the phenomena observed by Schreier and co-workers (6, 7) are not mediated by our Hf.

In our opinion, this discrepancy and others between our findings and those of the above investigators might be due to differences in methodology. Among these, we mention our use of affinity-enriched B cell cultures so that only 2×10^3 cells, with no requirement for additional filler cells, can mount a strong IgM response when

stimulated by appropriate factors. Another difference arises from the difficulty in determining which classes of cells are elaborating the various factors produced in their system (6, 7). It is thus possible that the characteristics attributed to antigen-specific T cell help (6, 7, 22, 44) might be due in part to the generation of nonspecific T cell factors as well as factors arising from sources other than T cells. In this regard, we stress that our current experiments using Hf, which is secreted by long-term cultured helper T cell lines without the use of filler cells and then purified by affinity-column chromatography, have so far yielded results that are similar to those reported here for Hf obtained by extraction (manuscript in preparation). It is also hoped that the approach of using better characterized factors, such as IL-1 or IL-2, in the place of various supernatant preparations will clarify some of the above-mentioned ambiguities.

We have previously shown (3) that Hf is allogeneically restricted in its cooperation with B cells. This restriction is controlled by the MHC region, and we are currently mapping it to specific MHC subregions. The requirement for histocompatibility between Hf and B cells for optimal interaction agrees with the concept of I-region-controlled allogeneic restriction between cooperating T and B cells (27). It is also compatible with the serological evidence by ourselves and others (45) for the presence of I-A determinants on Hf. Whether or not this determinant is identical with the interaction site for a corresponding B cell surface receptor remains speculative. We observe that, although allogeneic restriction is present when there is incompatibility between Hf and B cells, it is further enhanced if Hf is incompatible with both A cells and B cells. The parameters that affect this interaction are currently being analyzed; nevertheless, the data suggest that Hf-B cell interaction might involve events that are more complex than merely the binding of Hf to the B cell. The involvement of A cells at the level of MHC-controlled T-B cell cooperation is also borne out in other *in vivo* work from this laboratory (46).

In conclusion, we demonstrate that the proliferative and differentiative signals required for B cell triggering can readily be dissected. Antigen alone induces neither B cell proliferation nor differentiation into AFC. B cell proliferation is induced in the presence of Hf and antigen, but the reconstitution of a fully differentiated AFC response requires the additional presence of A cells or their secreted products. In the light of this and of our previous work, we would propose a tentative model for the events leading to antigen-specific B cell triggering as follows: antigen-primed helper T cells elaborate various factors, one of which is Hf, an antigen-specific Ia-bearing factor with a minimum 50,000 mol wt (5). This factor is specific, both in terms of its binding to antigen, and in terms of its functional activity in inducing a humoral response (2-5). Focused by its antigen-specific receptor to the antigen-binding B cell, Hf interacts in an H-2-restricted fashion with the cell surface membrane to induce the cell to proliferate. The mechanism of interaction between Hf and the B cell surface is unknown, but its control by the MHC raises the possibility that the I-A determinants found on Hf might be critically involved; whether or not the proliferative signal requires that Hf interact simultaneously with the Ig receptor-antigen complex and with other determinants on the cell surface membrane has not yet been determined. Differentiation into an IgM-secreting cell does not occur unless additional, nonantigen-specific signals are given by A cells. These A cells secrete soluble products, which, in the presence of Hf, promote the differentiation of B cells into AFC. However, in

the absence of Hf, B cells, even those that have bound antigen, are not receptive to this differentiative signal. The signal originates from a radioresistant cell population that includes Thy-1.2-bearing cells; the contribution of Thy-1.2-negative A cells in this process is not yet defined. IL-2 cannot by itself substitute for the differentiative signal in the Hf-mediated B cell response; if it is involved, it cannot be the only nonspecific signal required. The participation of A cells in the collaborative interaction with Hf and B cells also appears to be under MHC control but the precise cellular and molecular mechanisms by which this is done remain to be elucidated.

Summary

Proliferative and differentiative signals controlling the *in vitro* IgM response by unprimed, affinity-enriched B cells were studied using conditions under which as few as 2,000 B cells stimulated by antigen-specific, Ia-positive, allogeneically restricted, T cell-derived helper factor (Hf) or the polyclonal activator lipopolysaccharide (LPS) yielded on the average 400 antibody-forming cells (AFC) by direct plaque assay. Antigen alone induces neither B cell proliferation nor differentiation into AFC. Proliferation but not differentiation into AFC is induced when affinity-enriched B cells are cultured in the presence of Ag and Hf or LPS but in the absence of nonantigen-specific, radioresistant, accessory (A) cells. For the induction of a complete Hf- or LPS-mediated AFC response, cultures must be reconstituted with A cells or the secretory product(s) of these cells. The antigen-specific response depends strictly on the presence of the Hf specific for the relevant antigen, regardless of the cell cycle state of cooperating B cells. The differentiative signal from A cells is due, at least in part, to the presence of a Thy-1.2-bearing population of cells. In the case of the LPS-mediated, but not the Hf-mediated response, A cells can be substituted by using supernatant derived from an interleukin 2-secreting T lymphoma cell line (EL4). In the presence of histocompatible Hf and B cells, histoincompatible A cells can still cooperate in the immune response. However, the degree of allogeneic restriction between incompatible Hf and B cells is markedly increased if both B cells and A cells are incompatible with Hf.

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