REQUIREMENT FOR THREE SIGNALS IN "T-INDEPENDENT" (LIPOPOLYSACCHARIDE-INDUCED) AS WELL AS IN T-DEPENDENT B CELL RESPONSES*

By RUDOLF H. ZUBLER and ANDREW L. GLASEBROOK

From the Department of Immunology, Swiss Institute for Experimental Cancer Research, 1066 Epalinges, Switzerland

It has recently been reported (1–3) that at least three different activation signals are required by resting B lymphocytes to generate clones of antibody-secreting cells during a T helper cell (T_H)[1]-dependent humoral immune response: one signal is generated when B cells "see" antigen, another one when the T_H recognize major histocompatibility complex (MHC) (I region) determinants on the B cell. These two early signals are apparently required to render the B cell responsive to a third signal provided by T cell-derived nonspecific helper factor(s) for B cell responses (BHF). Thus, T_H provide two different types of B cell activation signals, one MHC-carrier-specific and one nonspecific (1–6). But it is still not clear whether an antigen (hapten)-B cell interaction generates by itself an activation signal or serves only to focus hapten-linked carrier determinants (and thus T help) onto the B cell (4, 7). In addition, it is not clear at the present time whether different factors (interleukins) are involved in B cell proliferation vs. differentiation (8–15) and whether the MHC-carrier-specific T_H-signal can be mediated by soluble T cell products (16–18). There is still conflicting evidence concerning the capacity of combinations of interleukins, e.g., concanavalin A (Con A) supernatants or secondary mixed leukocyte culture supernatants (MLC SN), to entirely substitute for T_H in T cell-depleted B cell cultures (8–15, 19, 20).

If there is a requirement for at least three signals in T_H-dependent B cell responses, one may also have to reconsider the hypothesis that a mitogen such as bacterial lipopolysaccharide (LPS) can bypass all three signals and by itself induce a full B cell response (3, 21). Different authors have to some extent observed synergistic effects between LPS and antigen (22, 23) or T help (24, 25) or both (26) but not a strict requirement for three signals. A major problem in the study of B cell activation, in particular concerning the role of LPS or the capacity of BHF to act as T-replacing factor, resides in the fact that B cell populations used for such studies still contain T cells that can themselves provide signals, especially in high density cultures.

The purpose of the present study was thus to characterize the requirements for the

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[1] *Abbreviations used in this paper:* BHF, T cell-derived helper factor for B cell responses; DMEM, Dulbecco's modified Eagle's medium; EL-4 SN, supernatant derived from EL-4 cells; FCS, fetal calf serum; HGG, human gamma globulins; KLH, keyhole limpet hemocyanin; LDA, limiting dilution assay; LPS, gram negative bacterial lipopolysaccharide; MHC, major histocompatibility complex; MLC, mixed leukocyte culture; MLC SN, secondary in vitro mixed leukocyte culture supernatant; NMS, normal mouse serum; PEC, peritoneal exudate cells; PFC, plaque-forming cells; sIg^+, sIg^-, surface immunoglobulin positive and negative; SRBC, sheep erythrocytes; T_H, T helper cells; TNP, trinitrophenyl.

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generation of plaque-forming cell (PFC) responses by purified surface Ig-positive (B) cells when cultured at a low cell density to minimize the possible effects of contaminating T cells. Using this system we now demonstrate that not only in T_H-dependent but also in LPS-dependent (i.e., so-called T-independent) PFC responses the B cells have to receive at least three different signals: (*a*) a specific T_H signal that can be bypassed by an LPS signal, (*b*) an antigen signal, and (*c*) a nonspecific T_H signal provided by BHF that cannot be bypassed by LPS. BHF-containing supernatants were derived from different cloned T_H lines, from EL-4 thymoma cells (27), or from secondary MLC, and none of these BHF preparations had the capacity to bypass the specific T_H or LPS signals. Moreover, it is shown that BHF acts on B cell proliferation but is not required for differentiation into PFC, in contrast to widely accepted current hypothesis (3, 8, 11–13).

Materials and Methods

Mice. C57BL/6 (B6), CBA, BALB/c, DBA/2, and (BALB/c × B6)F_1 mice were obtained from the colonies maintained at the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland. A.TH, A.TL, B10.D2, B10.AQR, and B10.A(4R) congenic and recombinant inbred mice were obtained from OLAC Laboratories, England.

Antigens and Mitogens. Keyhole limpet hemocyanin (KLH) and human gamma globulins (HGG) were coupled with trinitrophenyl (TNP) hapten and used for the immunization of mice as described (20). SRBC were obtained from Dr. T. Vischer, Hôpital Beau-séjour, Geneva, Switzerland. *Escherichia coli* O55:B5, LPS, prepared by the Westphal technique, was obtained from Difco Laboratories, Detroit, MI. Identical results were obtained with three different batches of LPS. LPS was dissolved in sterile phosphate-buffered saline (5 mg/ml) and stored at −20°C.

Cell Separation Technique. Spleen cells were separated into surface immunoglobulin-positive (sIg^+) and -negative (sIg^-) cells by incubation at 4°C in polystyrene petri dishes coated with affinity-purified rabbit anti-mouse Ig antibodies according to the panning method of Mage et al. (28). The sIg^+ cells were repeatedly analyzed using the fluorescence-activated cell sorter (sIg, Thy-1) and were found to contain >95% B cells (analyses kindly performed by Dr. R. P. Sekaly and Dr. H. R. MacDonald, Epalinges). The sIg^+ cells were also found to contain <1% α naphthyl acetate esterase-positive cells (analyses kindly performed by D. Heuman, Epalinges).

B Cell Cultures. All cultures of sIg^+ cells were performed in Dulbecco's modified Eagle's medium (DMEM) supplemented with additional amino acids (29) and including 10 mM Hepes, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum (FCS) (lot 904788, Seromed, Munich, West Germany). Cultures of 1 ml were performed in 16-mm wells (cluster 24-plates, Costar Data Packaging, Cambridge, MA), the limiting dilution assay cultures of 150 μl were performed in 6.4-mm wells (cluster 96-plates, flat-bottomed, Costar Data Packaging), and all cultures were kept without feeding or rocking in a 5% CO_2 incubator (20).

Secondary In Vitro MLC SN. The preparation of MLC SN has been described (30). The MLC SN used in this study was obtained from either B6 anti-DBA/2 or BALB/c anti-DBA/2 MLC.

EL-4 Supernatant (EL-4 SN). Culture supernatant from EL-4 thymoma cells was prepared by culturing EL-4 cells (10^6/ml) in medium with FCS during 40 h in the presence of 10 ng/ml of phorbol-12-myristate-13-acetate (PMA) as described by Farrar et al. (27). (The EL-4 SN was kindly provided by Dr. H. R. MacDonald and R. Lees, Epalinges; the interleukin 2-producing EL-4 subline was originally obtained from Dr. W. L. Farrar, National Institutes of Health, Bethesda, MD.)

Long-Term T Cell Clones and Cloned T Cell-Supernatants

ALLOREACTIVE T CELLS. Cells from primary or secondary MLC were cloned by limiting dilution (0.2 cells/culture) and expanded and maintained by weekly passage in cultures containing irradiated stimulator cells and MLC SN as described elsewhere (31). One long-term T cell clone (AB19) derived from an A.TH anti-A.TL MLC proliferated in the presence of

stimulator cells derived from A.TL, B10.AQR, B10.A(4R), and CBA mice but not from A.TL, B6, and BALB/c mice. Thus, the only alloantigen shared among the effective stimulator strains was I-A^k (32). Two clones were derived from BALB/c anti-DBA/2 MLC (AD2 and AD5), and two others were derived from B6 anti-DBA/2 MLC (M5 and D3). These T cells all reacted against non-MHC alloantigens, probably against Mls^a. Except for clone D3, which proliferated weakly, all these clones proliferated strongly in the presence of alloantigens but in the absence of exogenous interleukin 2 (MLC SN).

HGG-SPECIFIC T CELLS. T (sIg^-) cells (2×10^6/ml) derived from HGG-primed CBA mice were first cultured for 1 wk in DMEM-0.5% normal mouse serum (NMS), in the presence of HGG (10 μg/ml) and were then recultured every week in DMEM-5% FCS by adding 5×10^4 cells into 1-ml cultures containing 5×10^6 irradiated spleen cells and 10 μg HGG (but no MLC SN). After 5 wk, clones were obtained by limiting dilution (3 cells/culture) in 200-μl cultures containing 10^6 irradiated spleen cells, 10 μg of HGG, and 10% MLC SN. Cultures were then expanded, and the clone CR13 used for this study could be maintained by weekly passage as before cloning, i.e., in the absence of MLC SN. Clone CR13 exhibited helper activity for a PFC response against TNP-coupled HGG by CBA but not by B10.D2 sIg^+ cells and not for an anti-TNP-KLH response.

T CELL SUPERNATANTS. To obtain BHF derived from the alloreactive cloned T cells, 10^5 irradiated T cells were cultured in a 1.5 ml vol in the presence of 5×10^6 irradiated and anti-Thy-1 plus complement-treated stimulator cells. After 24 h, the culture supernatants were obtained and filtered (0.22-μm pores). BHF was similarly derived from clone CR13, but by culturing 10^6 T cells and adding HGG (10 μg/ml) into the 24-h culture.

Assay for PFC. A slide modification of the Jerne hemolytic plaque assay was used to detect either direct (IgM) or indirect (IgG) PFC (33). For the assay of IgG PFC, the formation of direct plaques was inhibited by the addition of goat anti-mouse μ-chain antiserum (N. L. Cappel Laboratories, Cochranville, PA) at a final dilution of 1:900 into the assay mixture (33). Rabbit anti-mouse Ig antiserum was used in a second step to develop the indirect plaques. Sheep erythrocytes (SRBC) or TNP-coupled horse erythrocytes (34) were used as indicator cells for anti-SRBC and anti-TNP PFC responses, respectively. Total PFC levels were measured by the protein A-PFC assay of Gronowicz et al. (35), slightly modified as described (36). Protein A-SRBC were obtained using the $CrCl_3$ coupling procedure of Goding (37). Except when otherwise mentioned, the PFC responses were measured on day 5 of culture, and the actual numbers of PFC are shown, i.e., background responses were not subtracted from the results.

Results

LPS, SRBC, and BHF Are Required to Generate an Anti-SRBC PFC Response in Low-Density B Cell Cultures. It was previously found by ourselves and others (8–10, 13–15, 19, 20) that anti-SRBC PFC responses can occur in cultures in which T_H are apparently substituted for by BHF, e.g., Con A supernatant, MLC SN, or supernatants from cloned T_H. These responses occurred even after treatment of B (sIg^+) cells with monoclonal anti-Thy-1 antibody and complement (20). However, our present experiments demonstrate that when the number of sIg^+ cells is reduced to 2×10^5/1 ml culture, no significant anti-SRBC PFC response is generated in the presence of SRBC and either MLC SN or EL-4 SN. When LPS was added to such cultures, a 100-fold higher response occurred. On the other hand, LPS by itself or together with antigen did not induce a significant anti-SRBC PFC response (Figs. 1 and 2). Thus, BHF was required during an LPS-dependent anti-SRBC PFC response, but the enhancing effect of BHF depended strictly on the presence of the antigen.

Limiting Dilution Analysis (LDA) of the LPS-dependent Anti-SRBC PFC Response. Limiting numbers of sIg^+ cells were placed into 150-μl cultures containing LPS and various supplements (SRBC, MLC SN, and irradiated syngeneic spleen cells) individually or in combination to study (*a*) the requirement for accessory

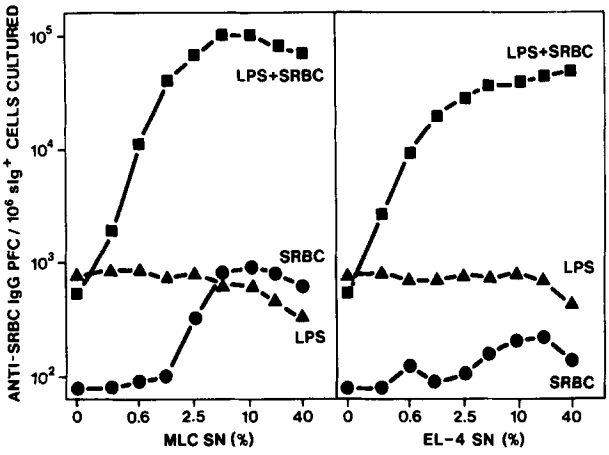

FIG. 1. Effects of MLC SN or EL-4 SN on the anti-SRBC IgG PFC response by unprimed B6 sIg^+ cells (2×10^5 cells/culture of 1 ml) in the presence of SRBC (●) (2×10^6 cells/ml) or LPS (▲) (50 μg/ml), or both (■). Less than 50 PFC were generated in the absence of SRBC and LPS.

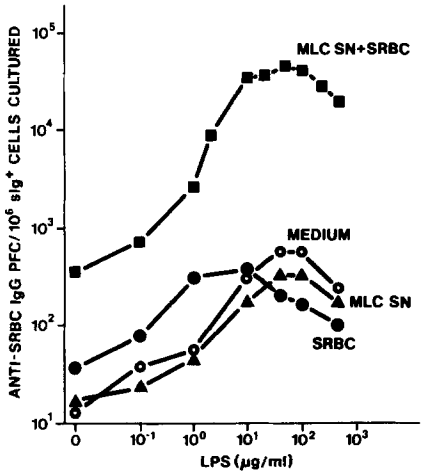

FIG. 2. Effects of LPS on the anti-SRBC IgG PFC response by unprimed B6 sIg^+ cells (2×10^5 cells/culture of 1 ml) in the presence of SRBC (●) (2×10^6 cells/ml), MLC SN (▲) (10%), SRBC and MLC SN together (■), or culture medium only (○).

(spleen) cells in LPS-dependent anti-SRBC PFC responses and (*b*) the clonal nature of such responses. As shown in Fig. 3, the generation of anti-SRBC PFC by 10^3 sIg^+ cells/culture required the presence of accessory cells, e.g., irradiated spleen cells (10^6/culture). T cell-depleted, irradiated spleen cells were also found to be effective, but splenic adherent cells alone were not (equivalent of 10^6 spleen cells/culture) (data not shown). Thus, it is not clear at the present time whether distinct accessory cells (macrophages [15]) or only a certain cell density (filler cell effect), or both, were required.

The results also demonstrate the generation of clonally expanded anti-SRBC PFC responses by 10^3 sIg^+ cells in cultures containing 10^6 irradiated spleen cells, LPS, SRBC, and MLC SN. When either MLC SN or SRBC were absent, LPS induced responses of only one or a few PFC/culture (Fig. 3). Poisson analysis of LDA data

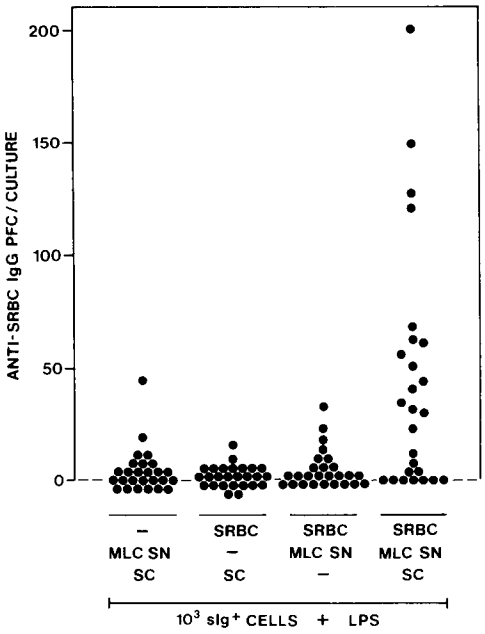

FIG. 3. Clonal LPS-dependent, anti-SRBC IgG PFC responses generated at a limiting concentration of sIg^+ cells/culture. Unprimed B6 sIg^+ cells (10^3) were added into 150-μl cultures in the presence of LPS (50 μg/ml), and, as indicated on the figure, SRBC (10^6 cells/culture), MLC SN (10%), and irradiated (2,000 rad) unseparated B6 spleen cells (SC). No PFC responses occurred in the absence of LPS.

(not shown) indicated that ~1/1500 B6 sIg^+ cells could generate a clone of anti-SRBC IgG PFC (>10 PFC/culture) in the presence of LPS, SRBC, and MLC SN, with a mean clone size of 45 PFC (range of 11–200 PFC). Thus, in the presence but not in the absence of BHF, clonally expanded, LPS-dependent anti-SRBC PFC responses did occur.

Effects of BHF on the Kinetics of the LPS-dependent Anti-SRBC PFC Response. The data presented so far did not show whether LPS by itself or together with antigen can induce the proliferation of antigen-specific B cell clones that do not, however, differentiate into PFC in the absence of BHF. If BHF is mainly a late-acting differentiation factor (8), one could expect that with a delayed addition of this factor into ongoing cultures containing sIg^+ cells, LPS and SRBC would either not change the kinetics of the PFC response or even allow for an enhanced clonal expansion of PFC precursors leading to higher PFC responses. However, as can be seen in Fig. 4, a delayed addition of MLC SN after 48 or 72 h changed the kinetics of the anti-SRBC IgM PFC response in such a way that the PFC appeared 24 or 48 h later, but their number was not increased. Moreover, the following observations were made of which results are not presented. (*a*) The IgG PFC responses were also retarded by the delayed addition of MLC SN, and EL-4 SN had the same effect as MLC SN. (*b*) A delay of 24 h in MLC SN addition had no significant effect on the kinetics of the anti-SRBC PFC response. (*c*) B cells could be washed in erythrocyte-lysing buffer (Tris-NH_4Cl) after they had been in contact with LPS and SRBC for 48 h and still generated a high anti-SRBC PFC response when cultured in the unique presence of

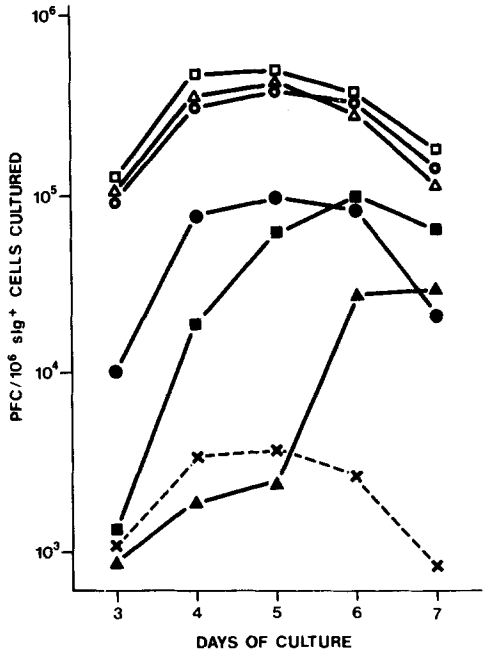

FIG. 4. Effects of a delayed addition of MLC SN on the kinetics of the LPS- and SRBC-dependent, anti-SRBC IgM PFC response (●, ■, ▲) by unprimed B6 sIg$^+$ cells: MLC SN (10%) was added either at the beginning of the cultures (●) or after 48 (■) or 72 (▲) h. The response that occurred in the absence of MLC SN is also shown (- - -). In addition, the protein A-PFC responses corresponding to the different times of MLC SN addition are shown (○, □, △). The 1-ml cultures contained 2 × 10^5 sIg$^+$ cells, 50 μg of LPS, and 2 × 10^6 SRBC. The PFC responses were measured on different days of culture.

TABLE I

Inhibition of the LPS-, SRBC-, and BHF-dependent Anti-SRBC PFC Response by Anti-SRBC Antiserum Requires the Early Presence of the Antiserum in the Culture

Culture conditions*	Anti-SRBC PFC/10^6 sIg$^+$ cells cultured	
	IgM	IgG
SRBC + MLC SN	250	100
LPS + SRBC	2,000	1,000
LPS + SRBC + MLC SN + NMS added on day 0‡	67,800	65,000
LPS + SRBC + MLC SN + B6 anti-SRBC serum added on day 0§	6,300	5,250
LPS + SRBC + MLC SN + B6 anti-SRBC serum added on day 2§	75,600	64,000

* The cultures (1 ml) contained 2 × 10^5 sIg$^+$ cells obtained from unprimed B6 mice and, where indicated on the table, LPS (50 μg/ml), SRBC (2 × 10^5/ml), and MLC SN (10%) were added on day 0.

‡ 0.5% normal B6 serum.

§ 0.5% serum from SRBC-primed B6 mice.

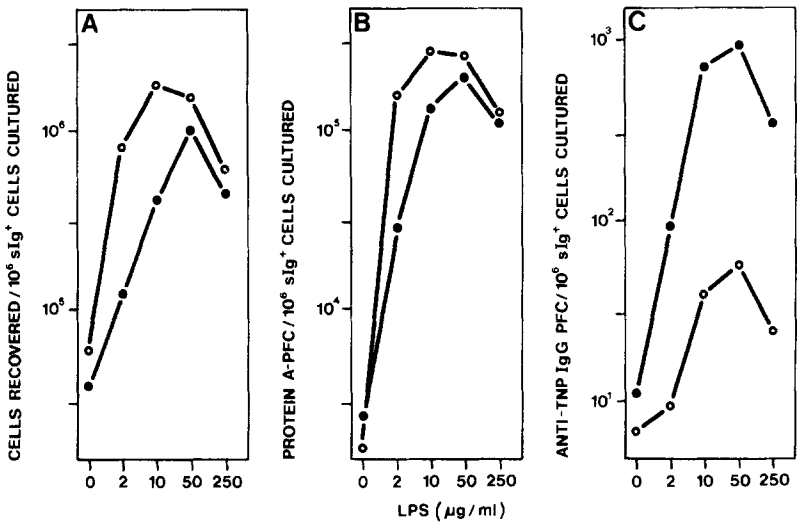

FIG. 5. Effects of LPS on the total cell recovery, the protein A-PFC response, and the anti-TNP IgG PFC response in the presence (●) or absence (○) of MLC SN (10%). The cultures (1 ml) contained 4×10^5 sIg^+ cells obtained from TNP-KLH-primed CBA mice and 100 ng of TNP-HGG and lasted 5 d.

MLC SN. (*d*) As shown in Table I, anti-SRBC antiserum, which inhibited the response by >90% when added on day 0 into the cultures (probably via masking of antigen [36]), had no inhibitory effect when added after 48 h. The results thus suggest that the SRBC-specific PFC precursors do not significantly proliferate in the absence of BHF and that LPS and antigen have to act for ~24 h on the B cell to render it responsive to BHF and are then no longer required.

Analysis of the Polyclonal LPS-dependent B Cell Response. It is also shown in Fig. 4 (see above) that 4×10^5 to 5×10^5 nonspecific protein A-PFC per 10^6 sIg^+ cells were generated on days 4 and 5 in cultures containing LPS and sIg^+ cells (2×10^5 cells/ml). The kinetics of this protein A-PFC response was not changed upon delayed addition of MLC SN. In cultures containing 4×10^5 sIg^+ cells/ml, the addition of LPS led on day 5 to a total recovery of about twice as many cells as initially added (Fig. 5). About 1 out of 10 recovered cells was a protein A-PFC. The cell recovery and the protein A-PFC response were not increased but slightly reduced in cultures containing 10% MLC SN in addition to LPS. However, in cultures containing TNP-HGG, the LPS-dependent anti-TNP IgG PFC response by TNP-primed sIg^+ cells was increased >10 times in the presence as compared to the absence of MLC SN. In another experiment (not shown), the anti-TNP IgG PFC response occurring in the presence of LPS (50 μg/ml) and TNP-coupled SRBC (10^5 cells/ml) was 78 times higher (17,400 anti-TNP IgG PFC/10^6 sIg^+ cells cultured) in the presence as compared to the absence of MLC SN. Thus, in contrast to these specific responses, the polyclonal B cell response induced by LPS in the absence of antigen was independent of BHF. However, LDA of the protein-A PFC response (not shown) indicated that about one out of three sIg^+ cells could generate one or a few (usually less than five) protein A-PFC after 5 d of culture in the presence of LPS, i.e., the protein A-PFC response involved no significant clonal expansion.

Effects of BHF Derived from Long-Term T Cell Clones. Two different long-term T_H

TABLE II

Effects of Cloned T_H of A.TH Origin Reacting against I-A^k and of T Cell Supernatant Thereof ($T_{H\text{-}IA}$ SN) on the Anti-TNP-KLH IgG PFC Response by CBA sIg^+ Cells

Culture conditions*	Anti-TNP IgG PFC/10^6 sIg^+ cells cultured	
	50 ng TNP-KLH/ml	50 ng TNP-KLH + 20 μg LPS/ml
2×10^5 $T_{H\text{-}IA}$ + 2×10^4 PEC	802	
6×10^4 $T_{H\text{-}IA}$ + 2×10^4 PEC	297	
2×10^4 $T_{H\text{-}IA}$ + 2×10^4 PEC	50	
2×10^4 PEC	<10	
6×10^4 $T_{H\text{-}IA}$ + 10% MLC SN	1620	
2×10^4 $T_{H\text{-}IA}$ + 10% MLC SN	612	
10% MLC SN	<10	1100
20% $T_{H\text{-}IA}$ SN‡	<10	345
20% control SN‡	<10	32
10% EL4 SN	<10	1020

* The cultures (1 ml) contained 4×10^5 sIg^+ cells obtained from TNP-HGG-primed CBA mice and the indicated concentrations of T_H and irradiated (2,000 rad) peritoneal exudate cells (PEC) or T_H and MLC SN or T cell supernatants. PEC were not required in cultures containing T_H and MLC SN.

‡ T cell supernatant and control supernatant from T cell-depleted stimulator cells were obtained as described in Materials and Methods.

TABLE III

Effects of Cloned T_H of CBA Origin Reacting against HGG and of T Cell Supernatant Thereof ($T_{H\text{-}HGG}$ SN) on the Anti-TNP-HGG IgG PFC Response by CBA sIg^+ Cells

Culture conditions*	Anti-TNP IgG PFC/10^6 sIg^+ cells cultured	
	50 ng TNP-HGG/ml	50 ng TNP-HGG + 20 μg LPS/ml
10^5 $T_{H\text{-}HGG}$ + 2×10^4 PEC	2916	
3×10^4 $T_{H\text{-}HGG}$ + 2×10^4 PEC	2160	
3×10^3 $T_{H\text{-}HGG}$ + 2×10^4 PEC	60	
2×10^4 PEC	<10	
3×10^4 $T_{H\text{-}HGG}$ + 10% MLC SN	3552	
3×10^3 $T_{H\text{-}HGG}$ + 10% MLC SN	792	
10% MLC SN	<10	980
20% $T_{H\text{-}HGG}$ SN‡	<10	396
20% control SN‡	<10	24

* The cultures (1 ml) contained 4×10^5 sIg^+ cells obtained from TNP-KLH-primed CBA mice and the indicated concentrations of T_H and irradiated PEC, T_H and MLC SN, or T cell supernatants.

‡ See legend to Table II.

TABLE IV

BHF Derived from Cloned Alloreactive T Cells is Only Active on sIg$^+$ Cells in the Presence of LPS

Origin of T cell supernatants*	Percent supernatant	Anti-TNP IgG PFC/10^6 sIg$^+$ cells culture‡	
		100 ng TNP HGG/ml	100 ng TNP HGG + 50 μg LPS/ml
No supernatant added	—	<10	206
MLC SN control	10	20	2757
SN clone AD2 (BALB/c anti-DBA/2)	20	<10	1831
	6	33	373
SN clone AD5 (BALB/c anti-DBA/2)	20	<10	1918
	6	40	160
SN clone M5 (B6 anti-DBA/2)	20	<10	1419
	6	120	420
SN clone D3 (B6 anti-DBA/2)	20	<10	313
	6	<10	220

* T cell SN were obtained from four clones reacting against non-MHC alloantigens (probably Mls^a) as described in Materials and Methods.

‡ The test cultures (1 ml) contained 3×10^5 sIg$^+$ cells obtained from TNP-KLH-primed (BALB/c × B6)F_1 mice.

TABLE V

Effects of Antigen and Exogenous BHF on PFC Responses Induced by Alloreactive (I-A^k-specific) Cloned T_H

Culture conditions*	PFC/10^6 sIg$^+$ cells cultured‡				
	No antigen	100 ng TNP-KLH/ml		2 × 10^6 SRBC/ml	
	Protein A-PFC	Anti-TNP IgG PFC	Anti-SRBC IgG PFC	Anti-TNP IgG PFC	Anti-SRBC IgG PFC
Control (medium)	2,400	<10	<10	<10	<10
10^5 $T_{H\text{-}IA}$	151,000	100	344	36	756
3×10^4 $T_{H\text{-}IA}$	69,000	72	32	28	244
10^5 $T_{H\text{-}IA}$ + MLC SN	158,000	1,028	336	100	1,900
3×10^4 $T_{H\text{-}IA}$ + MLC SN	88,000	340	60	32	1,500
50 μg LPS + MLC SN	274,000	1,740	312	184	6,200
MLC SN	2,640	28	<10	<10	<10

* The cultures (1 ml) contained 4×10^5 sIg$^+$ cells obtained from TNP-HGG-primed CBA mice and T_H (clone AB19) or LPS and/or MLC SN (10%), as indicated.

‡ In addition, the cultures contained TNP-KLH or SRBC as indicated, and both anti-TNP or anti-SRBC IgG PFC responses were measured in each type of cultures to control for possible nonspecific mitogenic effects of the antigens.

clones, one alloreactive specific for I-A^k and one HGG specific, were tested for helper activity in a hapten (TNP)-specific IgG PFC response by sIg$^+$ cells. As shown in Tables II and III, both were active. However, the cell-free culture supernatants derived from these T_H clones could not substitute for the presence of the T_H, although helper activity of the supernatants was detected in the presence of LPS. The effects of four other clone-derived T cell supernatants on an LPS- and TNP-HGG-dependent anti-TNP IgG PFC response are shown in Table IV. These supernatants were

obtained from T cells proliferating in response to non-MHC alloantigens (probably Mls^a determinants). The supernatants derived from the three clones that could proliferate in the absence of exogenous interleukin 2 (see Materials and Methods) exhibited BHF activity. Supernatants derived from a variety of T cell clones are currently studied in our laboratory with regard to different biological activities. None of the supernatants tested had a helper effect on the anti-SRBC PFC response by sIg^+ cells (2×10^5 cells/ml) except in the presence of LPS, and none of them have shown an MHC- and/or antigen-specific activity so far.

The Role of Antigen (Hapten) in B Cell Activation. It has been argued by some authors (4, 7) that an antigen signal is not required during B cell activation, but the results shown in Table V indicate that in LPS- as well as T_H-dependent B cell responses, antigen is required. Because the T_H in this case react against I-A on the B cell, antigen (hapten) is not required for focusing carrier determinants onto the B cells, but it must have some other effect. However, like LPS, the I-A^k-reactive T_H could induce background PFC responses against TNP or SRBC and a protein A-PFC response in the apparent absence of antigen. The T_H-induced responses against TNP or SRBC could only be enhanced by further addition of exogenous BHF (MLC SN) when antigen was present. The protein A-PFC response could not be enhanced by MLC SN (Table V).

Discussion

This report documents that in an LPS-dependent B cell response at least three different activation signals are required by resting B lymphocytes to generate expanded clones of PFC, namely (*a*) an LPS-signal, (*b*) an antigen signal, and (*c*) a signal provided by MHC- and antigen-nonspecific BHF. By use of highly enriched sIg^+ cells and by culturing these cells at lower density than that used in previous studies (21–26), it was possible to minimize the effects of residual T cells and thus to distinguish more clearly the different B cell activation steps. A close analogy was now found between LPS- and T_H-dependent B cell responses. T_H-dependent B cell responses require (*a*) a specific T_H-signal that is generated upon MHC (Ia)-restricted interaction with an activated T_H; (*b*) an antigen-signal that is generated when the B cells specifically recognize antigen (hapten) via surface Ig; and (*c*) a nonspecific T_H-signal that is provided by the BHF (1–3). It is shown for a response involving cloned allo-I-A^k-specific T_H that hapten recognition by the B cell itself generates an essential activation signal, i.e., that hapten binding serves not only to focus carrier determinants, and thus T_H, onto the B cell (4, 7). In addition, it is shown that two different T_H-signals are involved in B cell activation (1–6), i.e., that the effect of cloned T_H could not be fully substituted for by their own BHF. BHF derived from cloned T_H, EL-4 thymoma cells, or MLC could only act on sIg^+ cells in the presence of antigen and LPS or could enhance the effects of but not replace T_H. This was also true for anti-SRBC PFC responses previously thought to represent an exception (19, 20). The present data suggest that the LPS signal bypasses the specific T_H signal but only this signal.

With regard to the possible functions of the different signals involved in B cell responses, the key observations made in this study are the following: (*a*) Contact of sIg^+ cells with LPS alone is shown to lead to the generation of a polyclonal PFC response, but this response was associated with very limited proliferation. Previously,

a more significant effect of LPS on B cell proliferation was described in cultures containing also heterologous (rat) thymocytes (38) that most likely participated in B cell stimulation. Those results can thus not be compared with the present finding. In fact, B cell activation by LPS in limiting dilution cultures requires the presence of either filler cells (see Fig. 3) or additional mitogens, such as dextran sulfate as well as FCS and 2-mercaptoethanol (39–41), and thus it is not clear to what extent these various components contribute to the LPS effect. Clearly, LPS-induced background PFC responses were not increased by BHF in the absence of antigen.

(*b*) Similar to LPS, I-A-specific allo-T_H induced the generation of polyclonal PFC responses that in the absence of antigen were not increased by further addition of exogenous BHF. This independence of the protein A-PFC response from exogenous BHF, even at low T_H concentration, suggests that the specific T_H signal by itself can, like LPS, induce differentiation.

(*c*) Contact of sIg^+ cells with BHF alone, including BHF derived from MLC, had no effect. The protein A-PFC response was not increased over that occuring in normal culture medium. Thus, our observations differ from those made by others (42) in showing that no polyclonal differentiation of resting B lymphocytes was induced by BHF.

(*d*) Contact with antigen alone had no effect on B cell activation and did not induce BHF responsiveness. Anti-Ig reagents that were shown to induce BHF responsiveness by others (11, 12) might provide an additional signal to the one resulting from interaction with antigen receptors.

(*e*) Only in the presence of antigen and either LPS or a small number of T_H did the sIg^+ cells become responsive to BHF, after a lag period of ~24 h. Once the B cells had become responsive to BHF, LPS and antigen were apparently no longer required. When BHF (MLC SN or EL-4 SN) was added 48 or 72 h after LPS and antigen (SRBC), the appearance of PFC was delayed for 24 or 48 h. However, the delayed responses were not increased, as could have been the case if BHF were mainly a late-acting differentiation factor (8) and if, therefore, an extended proliferation phase had taken place in the absence of BHF. In contrast, this shows that BHF was required for significant clonal expansion to occur. Whether a differentiation enhancing component was also present in the BHF (3, 8–13) and whether such a component is in fact required is not known because the LPS-signal or the specific T_H signal (in the absence of which BHF activity could not be tested) induced differentiation by themselves.

Taken together, these observations support the following hypothesis of B cell activation: The LPS-signal or the specific T_H signal trigger the entering of resting (GO) B cells into the active cell cycle, leading to limited proliferation that is always associated with differentiation. The effect of BHF is then mainly to enhance proliferation, i.e., to act as a growth factor, and this effect can only occur when activated B cells have also received an antigen signal. The antigen signal is probably required for the expression or activation of BHF receptors on the B cells. Differentiation, on the other hand, appears to be a preprogrammed consequence of all B cell activation. It follows from this model that a humoral immune response induced by LPS or other polyclonal B cell activators that can bypass the specific T_H signal only involves significant clonal expansion when antigen (e.g., autoantigen [43]) and BHF are available. Responses against certain T-independent antigens (44)—or perhaps all such antigens—depend also on BHF for clonal expansion. However, B cell responses against

T-dependent antigens require in addition a specific T_H signal. Because T-B cooperation in vivo (45), and under certain conditions in vitro (6), depends on linkage between hapten and carrier determinants, the recognition of I region determinants on the B cell by the T_H requires most likely a simultaneous recognition of carrier antigen, i.e., the early T_H signal is both MHC- and carrier-specific. In a forthcoming report (manuscript in preparation) it will be shown that such a double restriction at the level of T-B interaction can occur. That is, in low-density cultures, male (H-Y) antigen-specific, long-term cultured T_H cooperate only with syngeneic male but not female B cells, even in the presence of male macrophages. It remains to be seen under which (apparently special) conditions soluble T cell products can mediate the specific T_H signal (16–18). Progress in this area should be most helpful in attempts to elucidate the nature of T cell receptor(s) for self and antigen.

Summary

The requirements for different activation signals in the generation of plaque-forming cell (PFC) responses by positively selected B (surface immunoglobulin-positive) cells were analyzed in low-density cultures to minimize the possible effects of contaminating T cells. Using this system, it is demonstrated that not only in T helper cell (T_H)-dependent but also in lipopolysaccharide (LPS)-dependent (i.e., so-called T-independent) PFC responses, the resting B cells have to receive at least three different signals: (*a*) a major histocompatibility complex (MHC)-specific T_H signal that can be bypassed by LPS, (*b*) an antigen signal, and (*c*) a second T_H signal mediated by MHC- and antigen-unspecific helper factor(s) for B cell responses (BHF) that cannot be bypassed by LPS. Specifically, contact of surface immunoglobulin-positive cells with cloned allo-I-A-specific T_H or LPS induced a polyclonal PFC response without significant proliferation, whereas contact with BHF alone (obtained as supernatants from different cloned T_H, EL-4 thymoma cells, or secondary mixed leukocyte culture cells) had no effect. Only when LPS, antigen, and BHF, or, alternatively, allo-T_H (producing themselves BHF) and antigen were present did clonally expanded PFC responses occur. Thus, the data indicate that both an LPS (or specific T_H) signal and an antigen signal are required to render the B cells responsive to BHF. BHF seems to act essentially as a nonspecific growth factor, whereas differentiation into antibody-secreting cells appears to be a preprogrammed consequence of B cell activation by an LPS or specific T_H signal.

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