REQUIREMENT FOR THREE SIGNALS IN B CELL RESPONSES

II. Analysis of Antigen- and Ia-restricted T Helper Cell-B Cell Interaction*

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It has recently been reported that resting B cells must receive at least three different signals to generate clones of antibody-producing cells in either a T helper cell (T_H)-dependent or a lipopolysaccharide (LPS)-induced (so called "T-independent") B cell response (1–3).[1] One signal (the specific T_H signal) is generated when activated T_H see Ia determinants (coded by the major histocompatibility complex) alone (1, 2) or together with antigen on the B cells (and it is this and only this signal that can be bypassed by LPS [3]); another is generated when B cells specifically see antigen (hapten) via surface immunoglobulin (sIg). These two signals are required to render the B cells responsive to a third (the nonspecific T_H) signal that is mediated by Ia or antigen-nonspecific B cell helper factor(s) (BHF). The exact nature of BHF is not yet known (4, 5) but this interleukin(s) is different from interleukin 2 (IL-2) (6, 7). Our own results have shown that BHF is essentially a B cell growth-promoting T_H product that enhances the clone size in plaque-forming cell (PFC) responses, whereas the differentiation of B cells into PFC appears to be a preprogrammed consequence of the specific T_H (or LPS) signal alone (3).

It is still not clear whether antigen-specific T_H must see both Ia and antigen on the B cells to provide the first, specific T_H signal (8, 9), or, as reported by some authors (1, 2, 10), only Ia on the B cells and antigen on the macrophages ($M\phi$). Moreover, it remains to be seen whether in a system in which T_H recognition of both Ia plus antigen on the B cells can be demonstrated, B cell-antigen (hapten) interaction by itself generates an essential signal (1–3). Using cloned T_H, several authors (1, 2, 10, 11) have failed to find a requirement for linked hapten-carrier recognition in vitro in contrast to in vivo studies (12). The demonstration of linked recognition in vitro requires highly purified B cells (13). However, it has been suggested (14, 15), but not directly demonstrated, that bystander B cell responses can occur when the B cells nonspecifically bind and present relevant carrier antigens to the T_H. Antigen presentation by B cells can be more directly analyzed by artificially coupling the carrier antigens onto the B cells or by the use of T_H reacting against cell membrane antigens.

By using these approaches, it has been found that T_H must see the antigens on the B cells (16–19). So far this has not been demonstrated with cloned T_H. Moreover, it has been concluded from these studies (16–19) that the B cells themselves need not see an antigen. On the other hand, the importance of a hapten signal has been deduced from the study of bystander responses occurring in the presence of horse erythrocyte-specific T_H and both horse and sheep erythrocytes (1, 2, 10). Although the importance of Ia recognition in T_H-B cell interaction is now widely recognized (1–3, 8–15, 17–19), it has not been clearly established whether anti-Ia antibody can specifically interfere with T_H- vs. LPS-induced B cell activation. One report (18) has shown that T_H-as well as LPS-induced responses can be inhibited by monoclonal anti-Ia antibody, but it was not demonstrated that the antibody acted at the B cell level. Finally, putative soluble factors mediating the Ia-specific T_H signal (20) have not yet been detected in a system in which the nonspecific BHF can be shown to have no effect on resting B cells (3).

We have established a cloned line of male (H-Y) antigen-specific T_H of C57BL/6 (B6) origin to study these questions. It will be shown that H-Y-specific T_H must see both H-Y and Ia determinants on the B cells to provide the first specific T_H signal. This signal is interfered with by monoclonal anti-I-A^b antibody at the B cell level, is not mediated by detectable soluble factors, and can be bypassed by LPS, in which case anti-I-A^b antibody has no effect. However, significant clonal expansion of PFC precursors occurs only when the B cells also see an antigen (hapten). Our main conclusion is thus that recognition of self-MHC together with antigenic determinants is as essential in T_H-B cell interaction as it is, for instance, in T cell-macrophage or cytolytic T cell (T_C)-target cell interactions.

Materials and Methods

Mice.   B6 male and female mice were obtained from the colony maintained at the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland. B10.D2 male mice were obtained from OLAC Laboratories, Bicester, England.

Antigens and Mitogens.   Sheep erythrocytes (SRBC) were obtained from Dr. T. Vischer, Hôpital Beau-Séjour, Geneva, Switzerland. Keyhole limpet hemocyanin (KLH) and human gamma globulins (HGG) were coupled with trinitrophenyl (TNP) hapten and used for the immunization of mice as described (13). *Escherichia coli* 055 B5 LPS, prepared by the Westphal technique, was obtained from Difco Laboratories, Detroit, MI and concanavalin A (Con A) from Pharmacia Inc., Uppsala, Sweden.

Cell Separation Techniques.   Spleen cells were separated into sIg^+ and sIg^- cells by incubation at 4°C in polystyrene petri dishes coated with affinity-purified rabbit anti-mouse immunoglobulin (Ig) antibodies according to the panning method of Mage et al. (21). The sIg^+ cell fraction contained >95% B cells and <1% α-naphthyl acetate esterase-positive cells (3). This cell fraction, representing 25–30% of the total spleen cell population, was used as the B cell source in all the experiments described below. Nylon wool-nonadherent spleen cells were prepared according to the method of Julius et al. (22) and were treated with monoclonal rat IgM antibody against nonpolymorphic determinants of Lyt-2 (clone 3.168.81, kindly provided by Dr. F. W. Fitch, University of Chicago, Chicago, IL) and rabbit complement (Low-Tox-M, Cedarlane Laboratories, London, Ontario, Canada) as described (23).

Culture for PFC Responses.   All cultures were performed in Dulbecco's modified Eagle's medium (DME) supplemented with additional amino acids (24) 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 cluster-24 plates (Costar, Data Packaging, Cambridge, MA), cultures of 200-μl in cluster-96 flat-bottomed plates (Costar), and all cultures were kept without feeding or rocking in a 5% CO_2-incubator.

Splenic Adherent Cells (SAC). To culture sIg$^+$ cells in the presence of irradiated SAC, the culture plates were set up with 3 × 10^6 spleen cells (irradiated with 2,000 rad) in 1 ml of DME-5% FCS and placed for 2 h at 37°C. Thereafter, the nonadherent cells were collected, the remaining adherent cells (equivalent of 3 × 10^6 spleen cells) washed three times with medium, and the sIg$^+$ cells added.

Secondary In Vitro Mixed Leukocyte Culture Supernatant (MLC SN) and EL-4 Thymoma Cell Supernatant (EL-4 SN). BALB/c anti-DBA/2 MLC SN was prepared as described (25). EL-4 SN was kindly provided by Dr. H. R. MacDonald, Ludwig Institute for Cancer Research, Epalinges. The IL-2- and BHF-producing EL-4 subline was originally obtained from Dr. W. L. Farrar, National Institutes of Health.

Clonal Assay of H-Y-specific T$_H$. The basic principle of this assay has been described (13). Nylon wool-nonadherent spleen cells or the Lyt-2-negative fraction thereof were obtained from B6 female mice that had been immunized intraperitoneally with 3 × 10^7 male spleen cells 3–5 wk earlier. In the first step of the assay, the tested T cell populations were titrated into 150-μl cultures containing 10^6 irradiated (2,500 rad) male spleen cells in DME-5% FCS supplemented with 10% MLC SN. The culture period was 13 d. In the second step (assay for helper activity), two aliquots of 50 μl of each culture were directly transferred into two 1-ml cultures containing 3 × 10^5 sIg$^+$ cells obtained from either B6 male or female mice and SRBC (2 × 10^6). The anti-SRBC IgG PFC response was measured after 5 d.

Clonal Assay for H-Y-specific T$_C$. The tested cells were titrated into 200-μl cultures (cluster-96 round-bottomed plates) containing 10^6 irradiated male spleen cells in DME-10% FCS supplemented with 25% MLC SN (26). The culture period was 7 d. Specific cytolytic activity was then measured in microtest cultures and by using ^{51}Cr-labeled Con A blasts derived from B6 male or female spleen cells (27).

Long-Term T Cell Clones. To obtain continuously growing cell lines of H-Y-specific T$_H$, 50 μl of cell suspension from the cultures set up for the clonal T$_H$ assay (see above) were transferred on day 13 into 1-ml cultures containing 5 × 10^6 irradiated (2,500 rad) male spleen cells in DME-5% FCS supplemented with 10% MLC SN. One line exhibiting H-Y-specific helper activity and several lines with either cytolytic or H-Y-nonspecific helper or only proliferative activity could be maintained for >3 mo by reculturing the cells every 5 d as above. The H-Y-specific T$_H$ line (B7) was subcloned by limiting dilution (3 cells/well) and conserved by freezing in 10% dimethyl sulfoxide after a total period of 4 mo in culture. A cloned line of SRBC-specific T$_H$ has also been used in some experiments (13).

Supernatant from H-Y-specific T$_H$ (T$_{H-HY}$). 10^7 cloned T$_{H-HY}$ were cultured in 10 ml of DME-5% FCS containing 4 μg/ml of Con A in a tissue culture flask (3013, Falcon Labware, Oxnard, CA). After 24 h, the culture supernatant was obtained by centrifugation and filtered (0.22-μm pores). Control supernatant was prepared from cloned H-Y-specific cytolytic T cells (T$_{C-HY}$).

Monoclonal Anti-IA^b Antibody. The hybridoma line MRC OX3 secreting mouse IgG anti-rat Ia antibody crossreacting with I-A^b (but not other H-2^b determinants, H-2^k, or H-2^d) (28) was kindly provided by Dr. A. F. Williams, Medical Research Council Cellular Immunology Unit, University of Oxford, Oxford, England. This line was used for ascites production in BALB/c mice.

Assay for PFC. A slide modification of the Jerne hemolytic plaque assay (29) was used to detect either direct (IgM) or indirect (IgG) anti-SRBC or anti-TNP PFC as described (3). Total PFC levels were measured by the protein A-PFC assay of Gronowicz et al. (30), slightly modified as described (23). PFC responses were measured by day 5 of culture, and the actual numbers of PFC are shown, i.e., background responses were not subtracted from the results.

Results

Clonal Assay for T$_{H-HY}$. A limiting dilution system was used to generate short-term clones of T$_{H-HY}$, and attempts were made to expand and maintain such clones as continuously growing T$_H$ lines. Thus, limiting numbers of nylon wool-nonadherent spleen cells obtained from B6 female mice primed with B6 male cells were titrated into cultures containing irradiated male spleen cells as a source of antigen and MLC SN as a source of T cell growth factor (see Materials and Methods). After 13 d the

cells from the individual cultures were tested for their capacity to provide help in anti-SRBC PFC responses of either male or female unprimed sIg^+ cells. As previously reported (3), it was important to use test cultures containing only 3×10^5 sIg^+ cells/ml to detect specific T_H-B cell interaction during anti-SRBC PFC responses. Such a reduced sIg^+ cell concentration apparently eliminates the possible participation in the anti-SRBC PFC response of T cells contaminating the B cell fraction. Moreover, as shown in Fig. 1, it was also important to treat the splenic (responder) T cell fraction with monoclonal anti-Lyt-2 antibody plus complement before the addition into the limiting dilution cultures. It was in fact found that ~1 out of 6,700 Lyt-2-negative male-primed splenic T cells could generate a clone of $T_{H\text{-}HY}$, whereas such clones were very rarely obtained with untreated T cells. This correlated with the presence of $T_{C\text{-}HY}$ precursors (~1 out of 4,500) in untreated T cells and their virtual absence in anti-Lyt-2 plus complement-treated splenic T cells. The $T_{C\text{-}HY}$ were found to act as H-Y-specific suppressor cells in the helper assay (see below). It should be mentioned that we have so far failed to detect suppressor T cell clones of a noncytolytic type in this system (data not shown). The actual PFC responses generated in a typical helper assay are shown in Fig. 2. It can be seen that H-Y-nonspecific T_H clones were also detected.

H-2- and H-Y-restricted Cloned T_H Line. A $T_{H\text{-}HY}$ clone could be maintained, expanded, and subcloned (see Materials and Methods). The function and specificity of the cloned $T_{H\text{-}HY}$ were characterized. When $T_{H\text{-}HY}$ were added into cultures containing irradiated B6 male SAC as a source of antigen-presenting cells, SRBC, and sIg^+ cells, an anti-SRBC PFC response occurred with B6 male but not with B6 female or H-2-congenic B10.D2 male sIg^+ cells (Fig. 3). The $T_{H\text{-}HY}$ could also induce a protein A-PFC response in the apparent absence of antigen, and this response was
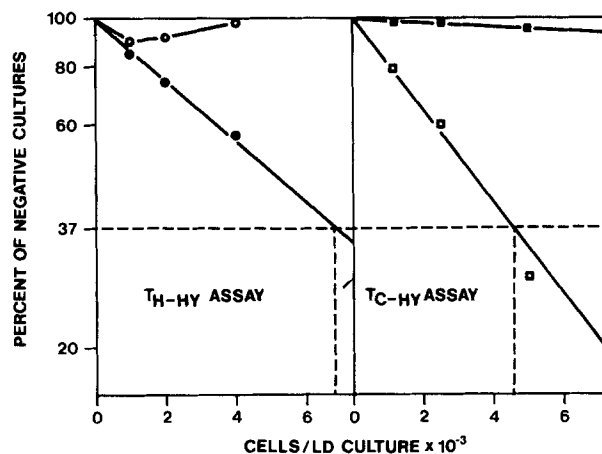

Fig. 1. Frequences of H-Y-specific T_H and T_C precursors in B6 female mice 3 wk after priming with 3×10^7 B6 male spleen cells. The limiting dilution cultures were performed as described in Materials and Methods by using either anti-Lyt-2 antibody plus complement-treated ($\bullet$, $\blacksquare$) or untreated ($\bigcirc$, $\square$) nylon wool-nonadherent spleen cells. Percentages of cultures that were negative for male-specific helper or cytolytic activity are plotted against numbers of cells added per limiting dilution culture (LD) (20 cultures/data point). According to Poisson statistics, 37% of negative cultures correspond to an average of one precursor per culture (27). (Helper or cytolytic activity in positive cultures exceeded the 2.5 SD range in control cultures without added T cells, and was >10-fold higher when assayed on male cells compared to female cells.)
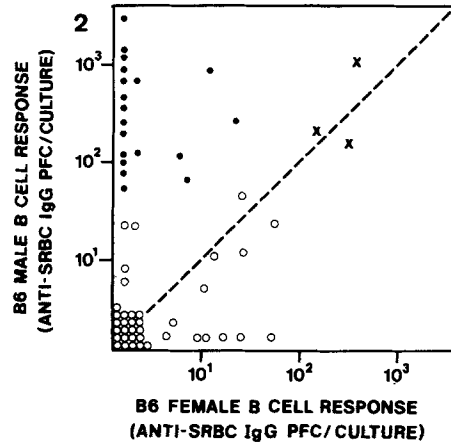
FIG. 2. Helper activity detected in individual limiting dilution cultures in a typical T_H assay (H-Y-specific [●], nonspecific [✗], or no [○] helper activity, same experiment as shown in Fig. 1). Three series of 20 cultures (150-μl vol, 10^6 irradiated male spleen cells, and 10% MLC SN) were set up with 1,000, 2,000, or 4,000 anti-Lyt-2 antibody plus complement-treated nylon wool-nonadherent spleen cells obtained from B6 female mice 3 wk after priming with male cells. After 13 d, 2 aliquots of 50 μl of each culture were directly transferred into two 1-ml cultures containing 3×10^5 sIg$^+$ cells obtained from either B6 male or female mice, and 2×10^6 SRBC. The anti-SRBC IgG PFC responses were measured 5 d later.
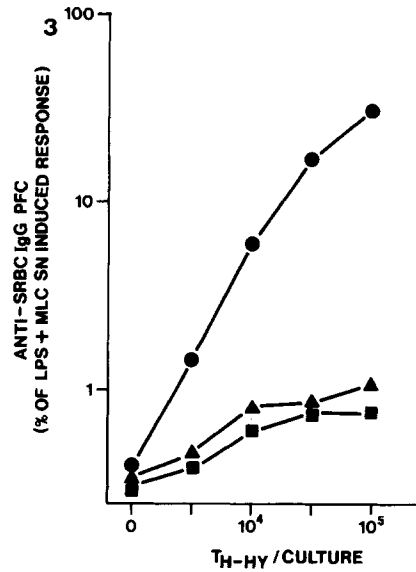
FIG. 3. H-2 and H-Y restricted T_H-B cell cooperation. Cloned B6 $T_{H\text{-}HY}$ were titrated into 1-ml cultures containing 3×10^5 sIg$^+$ cells obtained from either B6 (H-2^b) male (●), B6 female (■), or B10.D2 (H-2^d) male (▲) mice in addition to irradiated B6 male SAC (equivalent of 3×10^6 spleen cells) and SRBC (2×10^6). The anti-SRBC PFC responses generated are expressed as percentages of control responses occurring in the presence of 50 μg of LPS, 5% MLC SN, and SRBC (45,000–70,000 anti-SRBC IgG PFC/10^6 sIg$^+$ cells cultured).

also restricted to male as compared with ᶜ⁻male sIg⁺ cells, despite the presence of male SAC (Table I). Moreover, it was observed that in cultures containing a mixture of male and female sIg⁺ cells, SRBC, and $T_{H\text{-}HY}$, the generation of an anti-SRBC PFC response was inhibited by >90% in the presence of $T_{C\text{-}HY}$. This is compared with only 50% inhibition if the response in such cultures was induced by H-Y-nonspecific T_H or by LPS and MLC SN (Table II). These results demonstrate the high specificity of $T_{H\text{-}HY}$ (as well as that of $T_{C\text{-}HY}$). However, it is not known whether the T_C act (via lysis of antigen-presenting cells) mainly by preventing $T_{H\text{-}HY}$ activation. We are currently studying whether $T_{C\text{-}HY}$ can be used shortly before the PFC assay to type male and female PFC in mixed cultures.

$T_{H\text{-}HY}$ *Must See H-Y Determinants on the B Cells.*   Other experiments were performed to further study whether the specificity of $T_{H\text{-}HY}$ manifested itself at the level of T_H-B cell interaction or only during T_H-Mϕ interaction, i.e., whether fully activated $T_{H\text{-}HY}$ can cooperate with female B cells. The data presented in Table III demonstrate that the $T_{H\text{-}HY}$ became activated in the presence of irradiated male SAC, in that they could strongy enhance an LPS and SRBC-induced female anti-SRBC PFC response, i.e., could generate nonspecific BHF. Such activated T_H did not, however, induce a

TABLE I
Protein A-PFC Response Induced by H-Y-specific Cloned T_H

T_H added per culture	Protein A-PFC/10^6 sIg⁺ cells cultured*	
	B6 male sIg⁺ cells + B6 male SAC	B6 female sIg⁺ cells + B6 male SAC
10^5	87,800	3,200
3×10^4	39,200	1,960
10^4	18,800	1,600
3×10^3	10,400	800
0	1,640	1,280
LPS control‡	247,200	274,000

* The cultures (1 ml) contained 3×10^6 B6 male or female sIg⁺ cells in addition to irradiated B6 male SAC (equivalent of 3×10^6 spleen cells; see Materials and Methods).
‡ 50 µg/ml of LPS.

TABLE II
Specific Help and Suppression by Cloned $T_{H\text{-}HY}$ and $T_{C\text{-}HY}$

Culture conditions*	Anti-SRBC IgG PFC/10^6 sIg⁺ cells cultured		
	Male sIg⁺ cells	Male + female sIg⁺ cells	Female sIg⁺ cells
10^5 $T_{H\text{-}HY}$	14,100	8,000	120
10^5 $T_{H\text{-}HY}$ + 10^5 $T_{C\text{-}HY}$	350	620	<100
10^5 nonspecific T_H	37,000	19,100	25,300
10^5 nonspecific T_H + 10^5 $T_{C\text{-}HY}$	4,100	8,700	24,800
50 µg of LPS + 10% MLC SN	100,000	60,000	104,000
50 µg of LPS + 10% MLC SN + 10^5 $T_{C\text{-}HY}$	1,500	33,600	96,000

* The cultures (1 ml) contained 2×10^5 B6 male or female or 2×10^5 male and 2×10^5 female sIg⁺ cells in addition to 2×10^6 SRBC.

TABLE III

*T_{H-HY}, Which Become Activated in the Presence of Male SAC, Remain
Restricted to Cooperate with Male B Cells*

Culture conditions*	Anti-SRBC IgG PFC/10^6 sIg$^+$ cells cultured	
	Male sIg$^+$ cells	Female sIg$^+$ cells
T_{H-HY}	13,800	60
T_{H-HY} + LPS	20,400	1,620
LPS	1,840	1,460
T_{H-HY} + male SAC	16,200	960
T_{H-HY} + LPS + male SAC	30,800	33,600
LPS + male SAC	2,280	1,320
MLC SN + male SAC	260	380
MLC SN + LPS + male SAC	68,000	80,000

* T_{H-HY} (10^5) or MLC SN (5%), LPS (50 μg), and irradiated B6 male SAC
(equivalent of 3 × 10^6 spleen cells) were added into 1-ml cultures containing
2 × 10^5 B6 male or female sIg$^+$ cells in addition to 2 × 10^6 SRBC.

significant female response in the absence of LPS. This shows that the B cells must
themselves present H-Y antigen. The limited effect of T_{H-HY} on the female response
in the presence compared with the absence of male SAC could reflect an enhanced
proliferation of some already activated B cells in the presence of nonspecific BHF, as
a similar effect as with T_H was observed with MLC SN. It is also shown in Table III
that T_{H-HY} could induce a PFC response in male sIg$^+$ cells in the absence of SAC.
However, it is not known whether the B cells or some Mφ present in the sIg$^+$ cell
fraction activated the T_H, and to what extent the long-term cultured T_H were already
activated when used in these experiments.

*Monoclonal Anti-I-A^b Antibody Interferes with T_{H-HY}-induced but Not with LPS-induced B
Cell Activation.* It has been reported that B cell responses can be inhibited in vivo
(31) and in vitro (32) with anti-I-A or anti-I-E antibodies via an inhibition of T_H-Mφ
interaction. In another report (18) it was shown that T_H-induced as well as LPS-
induced B cell responses can be inhibited by anti-I-A antibody, but it was not
demonstrated whether the antibody acted at the B cell level. We have investigated
this point. In preliminary experiments, ascites containing a monoclonal anti-I-A^b
antibody was found to inhibit the proliferative responses of several cloned B6 anti-
H-Y T cell lines to B6 male spleen cells, but not that of B6 anti-DBA/2 (H-2^d) or
anti-AKR (H-2^k) alloreactive T cell lines to DBA/2 or AKR spleen cells (data not
shown). Therefore, the inhibitory effect on proliferation occurred at the level of the
stimulator cells. Next, it was found that the ascites containing anti-I-A^b antibody did
not inhibit an anti-SRBC PFC response generated in cultures containing B6 sIg$^+$ cells,
LPS, EL-4 SN (BHF), and SRBC, i.e., an LPS-induced B cell response (Fig. 4).
Moreover, the antibody was not by itself stimulatory for B cells (Fig. 4). It was thus
possible to study whether anti-I-A^b antibody interfered with T_{H-HY}-Mφ and/or T_{H-HY}-
B cell interaction. As shown in Table IV, ascites containing anti-I-A^b antibody
inhibited both the activation of T_{H-HY} in the presence of irradiated male spleen cells
leading to BHF generation (compare lines 3 and 4 in Table IV) and the induction of
an anti-SRBC PFC response by activated T_{H-HY} in male sIg$^+$ cells (compare lines 1
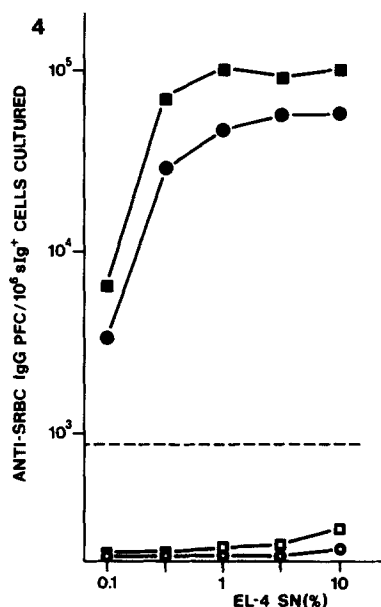
Fig. 4. Monoclonal anti-I-A^b antibody neither interferes with an LPS-induced B6 anti-SRBC PFC response nor induces a response in the absence of LPS. All cultures (1 ml) contained 2 × 10^6 B6 male sIg$^+$ cells, 2 × 10^6 SRBC, and EL-4 SN (different concentrations as shown). Shown are anti-SRBC IgG PFC responses in cultures with LPS (50 µg) and anti-I-A^b antibody (1% ascites) (■), LPS but no antibody (●), no LPS but antibody (□), or no LPS and no antibody (○). The LPS response observed in the absence of EL-4 SN is shown by the interrupted line.

TABLE IV

Monoclonal Anti-I-A^b Antibody Interferes with $T_{H\text{-}HY}$-Mϕ and $T_{H\text{-}HY}$-B Cell Interaction

Culture conditions*		Anti-SRBC IgG PFC/10^6 sIg$^+$ cells cultured	
Components added on day 0	Components added on day 1	Male sIg$^+$ cells	Female sIg$^+$ cells
$T_{H\text{-}HY}$ + SC	sIg$^+$ cells	12, 600	450
$T_{H\text{-}HY}$ + SC	sIg$^+$ cells + anti-I-A^b	50	500
$T_{H\text{-}HY}$ + SC	sIg$^+$ cells + LPS	NT‡	10,700
$T_{H\text{-}HY}$ + SC + anti-I-A^b	sIg$^+$ cells + LPS	NT	420
$T_{H\text{-}HY}$ + SC	sIg$^+$ cells + LPS + anti-I-A^b	NT	9,400

* The cultures (100 µl, 96-well plates) were initially set up with 2 × 10^5 irradiated $T_{H\text{-}HY}$ and 3 × 10^5 irradiated B6 male spleen cells (SC) in addition to 2 × 10^6 SRBC. After 24 h, 10^4 B6 male or female sIg$^+$ cells were added in a volume of 100 µl. Ascites containing monoclonal anti-I-A^b antibody (final concentration of 1%) and/or LPS (50 µg/ml) were added on day 0 or on day 1 as indicated.
‡ Not tested.

and 2). In this experiment, the sIg$^+$ cells were added 24 h later than the T_H and male spleen cells into the cultures. The culture conditions (see legend of Table IV) were those that we use in limiting dilution analysis of B cell responses (R. H. Zubler, manuscript in preparation). This assures a vast excess of activated T_H over B cells, but is not required to show inhibition by anti-I-A antibody. The data show that both T_H-Mϕ and T_H-B cell interactions were inhibited and thus involved recognition of

I-A^b determinants. On the other hand, blocking of Ia determinants was apparently of no consequence for the generation of an LPS signal.

Presence of Nonspecific and Absence of Specific Helper Factor(s) in T_{H-HY} Supernatant. The 24-h culture supernatant obtained from cloned T_{H-HY} (10^6/ml) stimulated with Con A (4 μg/ml) was tested for its capacity to mediate the specific and nonspecific helper functions of the T_H. As shown in Table V, this supernatant contained potent nonspecific BHF activity that enhanced an LPS- and SRBC-dependent anti-SRBC PFC response generated by either male or female sIg$^+$ cells. However, only a minimal enhancement of the anti-SRBC PFC response was detectable in the absence of LPS.

TABLE V

Nonspecific BHF Activity in Supernatant from Cloned T_{H-HY} (T_{H-HY} SN)

	PFC/10^6 sIg$^+$ cells cultured*			
	B6 male sIg$^+$ cells		B6 female sIg$^+$ cells	
Culture conditions	Anti-SRBC IgG PFC‡	Protein A PFC	Anti-SRBC IgG PFC‡	Protein A PFC
Control (medium)	<40	<500	<40	<500
T_{H-HY} SN (20%)§	784	1,000	464	3,400
LPS (50 μg)	376	164,000	900	193,000
LPS (50 μg) + T_{H-HY} SN (20%)	16,400	170,000	23,000	196,000

* The cultures (1 ml) contained 2 × 10^5 B6 male or female sIg$^+$ cells.
‡ 2 × 10^6 SRBC/culture.
§ Supernatant from Con A-stimulated T_{H-HY} (10^6/ml) was obtained as described in Materials and Methods.
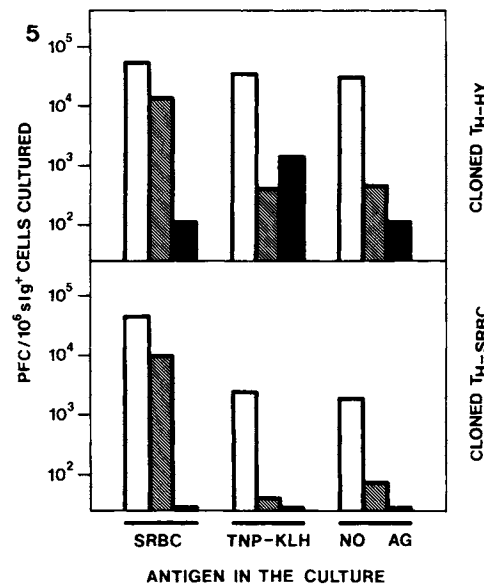

FIG. 5. Effects of two different antigens (SRBC or TNP-KLH) on PFC responses depending on cloned T_{H-HY} or T_{H-SRBC}. The T_H (10^5) were added into 1-ml cultures containing 2 × 10^5 sIg$^+$ cells obtained from TNP-HGG-primed B6 male mice. Either SRBC (2 × 10^6), TNP-KLH (100 ng) or no antigen was added. Protein A-PFC (□) responses and SRBC- (▨) or TNP-specific (■) IgG PFC responses are shown. (1,800 protein A-PFC and <20 anti-SRBC or anti-TNP IgG PFC were generated per 10^6 sIg$^+$ cells cultured in the absence of T_H).

This was not specific for the male response and occurred also with MLC SN (see above, Table III). Clearly, the T_{H-HY}-supernatant could not induce a protein A-PFC response (also shown in Table V), i.e., could not provide a polyclonal B cell inducer signal like T_{H-HY} or LPS.

Effect of Antigen (Hapten)-B Cell Interaction on the T_{H-HY}-induced PFC Response. The effects of two different antigens (SRBC and TNP-KLH) on the specific and protein A-PFC responses induced by either H-Y- or SRBC-specific cloned T_H were analyzed. As shown in Fig. 5, the helper activity of T_{H-HY} but not that of T_{H-SRBC} was polyclonal in nature, i.e., T_{H-HY} induced a high protein A-PFC response and significant anti-SRBC and anti-TNP PFC responses in the apparent absence of antigen in the culture. However, the anti-SRBC and anti-TNP PFC responses were increased >10 times in the presence compared with the absence of the corresponding antigens. By limiting dilution analysis it was found that B6 mice that are genetically high B cell responders against certain batches of SRBC (23) have ~600 anti-SRBC PFC precursors in 10^6 unprimed sIg^+ spleen cells (3) compared with only ~50 anti-TNP-(KLH) PFC precursors in 10^6 TNP-primed sIg^+ spleen cells (data not shown). Therefore, both specific PFC responses observed in the presence of T_{H-HY} and antigen (see Fig. 5) involve a considerable clonal expansion of the PFC precursors.

Discussion

We have established a cloned line of T_H of B6 female origin that specifically recognize male (H-Y) antigenic and I-A^b determinants on Mϕ and on B cells. The different variables that determine why only a small minority of short-term T_H clones can be maintained for long periods in culture are still largely unknown (33). However, because it is demonstrated that 1 in 6,700 Lyt-2-negative splenic T cells in B6 female mice primed with male cells could generate a short-term clone of H-Y-specific T_H, it appears that with regard to specificity, the long-term line does not represent an exception. The detection of T_{H-HY} in the total (Lyt-2-negative and -positive) splenic T cell population was to a high degree prevented by the presence of H-Y-specific T_C (1 in ~4,500 T cells) that acted as potent, specific "suppressor T cells" during the helper assay. B6 mice are known to generate strong H-Y-specific T_C as well as T_H responses (34). The efficiency of T_C in inhibiting B cell responses makes them ideal candidates for suppressor T cells in some in vivo antibody responses.

Certain T_H appear to recognize self Ia in the absence of an antigen, and this may be of physiological relevance (35). It is not known whether the H-Y-nonspecific T_H precursors detected in B6 female mice primed with male cells belong to this category of anti-self Ia reactive T_H or if they see self Ia together with autoantigens. In particular, the induction of a strong T_{H-HY} response as demonstrated above could favor the concomitant induction of an autoreactive T_H response. However, this report shows that T_H specific for self Ia plus H-Y antigen must see both antigen and Ia on all cells they interact with. In particular, they must recognize antigen and Ia on the B cells. In this regard the data obtained with cloned T_H confirm previous findings made with uncloned T cell populations reacting against cell membrane or cell-bound antigens (16–19). Thus, T_{H-HY} induced a PFC response in B6 male but not in B6 female or H-2-congenic B10.D2 male B cells. Such specificity was retained by already activated T_H and occurred at the level of T_H-B cell and not just T_H-Mϕ interaction, i.e., in cultures containing B6 male SAC and female sIg^+ cells, the T_{H-HY} generated BHF

and thereby strongly enhanced an LPS and SRBC-dependent anti-SRBC PFC response, despite their inability to induce the response by themselves. Moreover, it is also shown that a monoclonal antibody directed against I-A^b determinants interfered with both T_H-Mϕ interaction leading to T_H activation and activated T_H-B interaction leading to the induction of a PFC response. In contrast, an LPS-, antigen-, and BHF (EL-4 SN)-dependent PFC response was not inhibited by the anti-I-A^b antibody. The antibody did also not induce a response by itself in the absence of LPS. These results confirm that T_H must see Ia on the B cells and suggest that LPS must not react with Ia. Others have reported that a monoclonal anti-I-A antibody interfered with an LPS-induced B cell response (18). The discrepancy with our results could be explained by a difference between the monoclonal antibodies or by the fact that exogenous BHF was added in our experiments, and thus did not have to be provided by some T cells in the cultures.

By analogy to a T_{H-HY}-induced B cell response, one could hypothesize that all T_H recognizing self Ia plus antigen must see the antigen on the B cells, but this is difficut to prove for nonmembrane antigens (8, 9, 13–15). It has recently been shown that B lymphocyte tumor cells can present such antigens to proliferating T cells (36). However, it remains to be studied with cloned B cells (4) whether the B cells can produce the IL required for the T_H-activation, in particular IL-1 (37). With regard to the two signals that are provided to the B cells by the T_H (1–3, 19) it can now be stated that the first (specific) T_H signal is generated when the T_H see both antigen and Ia on the B cells, whereas the second (nonspecific) T_H signal is mediated by Ia and antigen-nonspecific BHF. In addition, B cell-antigen (hapten) interaction generates an essential signal (1–3). In a previous report (3) it was shown that the specific T_H signal provided by alloreactive T_H and an LPS signal are equivalent in inducing polyclonal PFC responses (i.e., B cell differentiation) in the absence of an antigen seen by B cells. This response, however, does not involve significant clonal expansion of the PFC precursors (i.e., proliferation). PFC responses induced by either the T_H or LPS could be enhanced by addition of exogenous BHF (MLC SN, EL-4 SN, or various cloned T_H supernatants), but this required the presence of an antigen seen by the B cells. Therefore, the antigen signal in addition to the specific T_H (or LPS) signal is required to render B cells responsive to the growth-promoting BHF. The results from this study show that B cell responses induced by H-Y-specific T_H are not different from those induced by alloreactive T_H with regard to the requirement of an antigen signal. This effect of antigen has not been considered during previous studies (16–19) with T_H reacting against B cell membrane or cell-bound antigens.

The molecular mechanisms involved in T_H-B cell interaction are not known. It would facilitate their investigation as well as that of T cell antigen/Ia receptors if soluble factors mediating the specific T_H signal could be obtained. The conditions used in this study for the detection of a putative specific helper factor generated by cloned T_{H-HY} were those required for the study of specific T_H-B cell interaction itself, i.e., low-density cultures containing positively selected sIg$^+$ cells and FCS that apparently has no LPS-like B cell activating activity. No significant protein A or anti-SRBC PFC response occurred in these cultures upon addition of a high concentration of BHF (e.g., MLC SN or EL-4 SN) and antigen (SRBC). It is shown that supernatant from Con A-stimulated T_{H-HY} induced neither a protein A-PFC response nor a significant anti-SRBC PFC response (in the presence of SRBC), although it exhibited

potent BHF activity. Similar results were previously obtained with supernatants from different alloreactive cloned T_H lines (3). It is difficult to compare these results with those obtained when putative specific T cell factors were detected under different assay conditions, e.g., in which BHF itself apparently induced PFC (20) or in which T cells were present (38). However, the possible requirement for cell-to-cell contact in T_H-B cell interactions, as it is required, for instance, in T_C-target cell interactions, should be considered in future investigations. Because we have found that not all I-A^b plus H-Y-specific proliferating T cells can provide a B cell activating signal (unpublished observation), it is possible that Ia recognition itself is not directly involved in B cell activation but rather in the activation of some helper machinery in the T cells.

In conclusion, our results thus show a requirement for direct Ia plus antigen-specific T_H-B cell interaction in addition to the lymphokine-mediated T_H function(s) in the anti-SRBC PFC response. This holds at least for the B cells recovered by the panning method (representing 25–30% of all spleen cells). In a previous study (13) we have shown that H-2-restricted T_H-B cell interaction is also required in secondary in vitro anti-TNP-KLH or anti-TNP-*Leishmania tropica* PFC responses. On the other hand, Asano et al. (39) have recently reported that one B cell subset characterized by the presence of the Lyb-5 differentiation antigen can respond to TNP-KLH in the absence of H-2-restricted T_H-B cell interaction. This B cell subset, however, responded only at a very high TNP-KLH concentration, at which it is difficult to rule out some T-independent antigen-like effects of TNP-KLH. This may be relevant, as the Lyb-5-negative B cells not responding in the absence of an H-2-restricted T_H signal are also those B cells which cannot respond to certain T-independent antigens. In any case, it is clear that the question of whether different subsets of resting B cells exhibit differential activation requirements is important and needs further investigation.

Summary

We have recently reported that resting B cells must receive at least three different signals in a T helper cell (T_H)-dependent as well as in a lipopolysaccharide (LPS)-induced B cell response (3), i.e., a specific T_H signal (that can be bypassed by LPS), a nonspecific T_H signal (mediated by Ia or antigen-nonspecific B cell helper factor), and an antigen (hapten) signal.

In a system using male (H-Y) antigen-specific cloned T_H of C57BL/6 origin and male (or female) B cells, we now confirm and extend these findings by demonstrating that H-Y-specific T_H must see both H-Y and Ia determinants on the B cells (and not only on macrophages) to provide the first specific T_H signal required for a plaque-forming cell (PFC) response. This signal was interfered with by a monoclonal anti-I-A^b antibody at the B cell level, was not mediated by detectable soluble factors (in contrast to the nonspecific signal also provided by the T_H), and could be bypassed by LPS, in which case anti-I-A^b antibody had no effect. However, although the H-Y-specific T_H induced a polyclonal PFC response (B cell differentiation) in the apparent absence of an antigen seen by the B cells, significant clonal expansion of PFC precursors occurred only when the B cells also recognized an antigen (hapten).

Note added in proof: The following papers have appeared since this manuscript had been written: Julius, M. H., H. von Boehmer, and C. L. Sidman. 1982. Dissociation of two signals required for activation of resting B cells. *Proc. Natl. Acad. Sci. U. S. A.*

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Julius et al., by using cloned T_{H-HY}, demonstrated a requirement for H-Y- and I-A-restricted T_H-B cell interaction and anti-Ig antibody for the activation of resting B cells. Pettersson et al., by using uncloned T_{H-HY}, concluded that only a specific T_H-B cell interaction, but no antigen (hapten) signal, is required. We believe that our own results are consistent with but more complete than these newly published data. We are in agreement with Julius et al. with regard to the specificity of T_H-B cell interaction. As shown by Pettersson et al., we find that T_{H-HY} alone induce a polyclonal PFC response. The clonal expansion of specific PFC precursors is, however, strongly dependent on an antigen signal and nonspecific BHF as a third signal. It is possible that Julius et al. did not detect a response in the absence of anti-Ig antibody because their cloned T_H had a lower helper activity than ours.

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