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T-cell-dependent B-cell stimulation is *H-2* restricted and antigen dependent only at the resting B-cell level

(*in vitro* sensitization/helper T cells/B-cell growth/H-2 preference/resting state of lymphocytes)

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ABSTRACT Cloned lines of helper thymus-derived (T) cells produce help for bone marrow-derived (B) cell growth and Ig secretion in the presence of histocompatible adherent cells and of specific antigen. This help stimulates histocompatible as well as histoincompatible B-cell blasts polyclonally. Thus, neither antigen nor histocompatibility, but antigen-unspecific factor(s) for growth and Ig secretion are required to stimulate a B-cell blast through the next round of division. On the other hand, only histocompatible, resting, small B cells, and only those binding their specific antigen, can be stimulated by antigen-activated T-cell help to initiate growth and Ig secretion. The preference of the resting B cells for such collaboration with T-cell help is mapped to the *K* end of the *H-2* histocompatibility loeus, and probably constitutes the antigen expressed on B cells by the immune response (*I*) region. It appears that a resting B cell is excited by the binding of specific antigen to surface Ig and by the interaction of its surface *Ia* antigen with helper T cells. After this dual recognition the excited B cell can be stimulated by the antigen-unspecific factor(s) generated by the interaction of helper T-cells, adherent cells, and antigen to initiate replication.

Thymus-derived (T) lymphocytes recognize antigen in the context of Ia determinants on macrophages (1). This leads to activation of the helper T cells and to production of helper factor(s) by either the activated helper T cells or the macrophages (2, 3). Clones of helper T cells require the presence of both the antigen and immune response (*I*) region-compatible macrophages to produce help for bone marrow-derived (B) cell stimulation (4).

Activated helper T cells also appear to interact with antigen and Ia determinants on B cells (5–8), though in other investigations (9) such interactions were found not to be H-2-restricted. If this interaction is to be H-2 restricted, B cells have to express Ig, to recognize antigen, and express Ia, to be recognized by T-cell help, in order to react by replication and Ig secretion.

We have recently found that activated B-cell blasts are stimulated by antigen-activated T-cell help—generated by antigen, specific helper T cells, and histocompatible macrophages—*polyclonally* and irrespective of their H-2 type (10). Such antigen-activated T-cell help, in fact, acts via soluble helper factor(s) that stimulate activated B-cell blasts to further replication and Ig secretion; however, the factor(s) induce small B cells only to maturation into Ig secretion without replication (unpublished data).

The polyclonal and H-2-unrestricted response of B-cell blasts is in apparent contrast to the specific and H-2-restricted response of normal B cells stimulated by T-cell-dependent antigens. Most lymphocytes are normally not in an activated blast state but are resting. This paper investigates the action of antigen-activated T-cell help on such resting small B cells.

MATERIALS AND METHODS

Animals. C57BL/6J/Fül *nu/nu* and normal mice, 6 to 8 weeks of age, and Lewis strain rats, 4 weeks of age, were obtained from the Institut für Biologisch-Medizinische Forschung, Füllinsdorf, Switzerland. C3H/Tif *nu/nu* and BALB/c *nu/nu* mice, 6–8 weeks of age, were from Gr. Bomholtgaard, Ry, Denmark. B10.A(4R) and B10.A(5R) mice, 2–4 months of age, were obtained from OLAC, Oxon, England.

Cell Cultures. Mouse spleen cells, prepared as described (11, 12), were cultured in Iscove's medium (13), containing transferrin, albumin, and soybean lipid as serum replacements and also 2-mercaptoethanol (50 μM) and kanamycin (Bio-Cult, Irvine, Scotland). Splenic B cells were activated by lipopolysaccharide S (LPS-S) [smooth form, a gift from C. Galanos and O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg i.Br., W. Germany (50 μg/ml)] in culture for 48 hr at 3×10^5 cells per ml (10, 14). Activated B-cell blasts were separated by velocity sedimentation at unit gravity (15) and collected as described (10, 14).

Cell concentrations covered the range of 3×10^5 to 1 B-cell blasts per culture in 1:3.3 dilution steps. Ten cultures for each dilution were set up in Falcon Microtest II plates (catalog no. 3040). According to Poisson's distribution one B-cell precursor for a clone of cells secreting a given type of Ig is present in the number of cells plated in individual cultures that lets 63% of all cultures appear positive in the assay. All frequency determinations in this paper, due to the rather wide 1:3.3 dilution steps and to the limited numbers of individual cultures assayed, must be regarded as approximate. Earlier more precise frequency determinations, of reactive B cells in similar limiting dilution experiments, supporting results of approximate determinations (16), justify such work-saving approximate determinations as made in this paper.

Helper T cells specific for horse erythrocytes (HRBC) (T'_{HRBC}) were obtained from irradiated, thymocyte-reconstituted, antigen-primed C57BL/6J/Fül mice (17). Spleen cells of these were used as *in vivo* activated helper T cells at 1×10^6 cells per ml after irradiation with 3300 rads (1 rad = 0.01 gray) with a Phillips RT 305 x-ray machine in experiments of Table 1. In all other experiments helper T cells were used that had been propagated *in vitro* for more than one year and cloned as described (4). Clone 18-19, which was used throughout the present studies, was titered for maximal helper activity and used as 5×10^3 cells per ml. B-cell blasts were enriched from mitogen-activated spleen cells, and small B cells were enriched from

Abbreviations: T, thymus-derived; B, bone marrow-derived; H-2, major histocompatibility complex in the mouse; I, immune response genes within *H-2* complex; Ia, antigens on certain cells (possibly coded for by the *I-A* subregion of the *H-2* complex); LPS, lipopolysaccharide; HRBC, horse erythrocytes; SRBC, sheep erythrocytes; TNP-SRBC, trinitrophenylated SRBC; T'_{HRBC}, T cells primed to HRBC; PFC, plaque-forming cells; F_B, growth-stimulating factor for B cells.

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normal fresh spleen cells at the same time by velocity sedimentation. The small B cells or the B blasts were then cultured at different concentrations in the presence or absence of homologous mitogen (LPS) in the presence of 3×10^6 rat thymus cells per ml, with or without antigen [HRBC or sheep erythrocytes (SRBC), 2.5×10^6 per ml] or in the presence of T'_{HRBC} cells, antigen, and 1×10^6 irradiated C57BL/6J *nu/nu* spleen cells per ml.

Anti-Thy-1.2 Treatment of Lymphocytes. The rat–mouse hybridoma J-1-j producing monoclonal anti-Thy-1.2 antibody (kindly given to us by J. Sprent, University of Pennsylvania, Philadelphia, PA) was grown in serum-substituted Iscove's modified Dulbecco's modified Eagle's medium (13). Splenic lymphocytes (10^7) were incubated for 30 min at 37°C in a mixture of 0.1 ml medium in which the hybridoma cells had been grown for 3 days to a density of $>2 \times 10^6$ cells per ml and 0.05 ml of guinea pig complement (GIBCO; Bio-Cult, Irvine, Scotland), previously absorbed twice with an equal volume of packed mouse splenic lymphocytes (C57BL/6J). The remaining cells were then washed three times with balanced salt solution and cultured.

Assays. Cultures were assayed for IgM-secreting cells by the protein A-SRBC plaque assay (18). B-cell clones secreting antibodies against SRBC, HRBC, or trinitrophenylated SRBC (TNP-SRBC; 30 mg of 2,4,6-trinitrobenzenesulfonic acid coupled to 1 ml of packed SRBC) were detected by the appropriate antigen-specific plaque assays (19). Proliferation was measured by a 2-hr pulse of 5 μCi/ml of [^{3}H]thymidine (2 Ci/mmol, Amersham, England; 1 Ci = 3.7×10^{10} becquerels) as described (13).

RESULTS

Stimulation of B blasts and of small B cells by HRBC-activated C57BL/6J T-cell help or by LPS

Thymidine Uptake and Development of IgM-Secreting Plaque-Forming Cells (PFC). Both resting small B-cells of C57BL/6J *nu/nu* spleen and C57BL/6J *nu/nu* splenic LPS-activated B-cell blasts could be stimulated by LPS to display increased thymidine uptake, clonal growth, and maturation to IgM-secreting PFC to levels and in frequencies expected from previous experiments (12, 14) (Table 1). In the absence of mitogen there was no response. Antigen-activated T-cell help (4, 10) on the other hand, acted differently on activated B blasts and on resting small B cells. In agreement with previous results (10), B-cell blasts were stimulated to increase thymidine uptake and to grow polyclonally and develop IgM-PFC both by antigen-activated T-cell help (T'_{HRBC} + HRBC) and by LPS. Resting small cells, however, showed no measurable increase in thymidine uptake. The development of IgM-PFC was nearly 1/10th of that with LPS; it is due to maturation of small resting B cells to IgM-PFC without replication (unpublished data). Restimulation of B-cell blasts by antigen-activated T-cell help could also be observed with "background" activated blasts, isolated from normal spleen by velocity sedimentation (refs. 14 and 15, data not shown). This rules out that antigen-activated T-cell help acts in an "adjuvant-like" action on LPS-coated B blasts only.

Thus antigen-activated T-cell help stimulates B blasts polyclonally to division and IgM secretion but induces small B cells only to IgM secretion without replication.

Frequencies of Growing Antigen-Specific B Cell Clones. The B-cell mitogen LPS triggered HRBC-, SRBC- and TNP-SRBC-specific B cells to clonal growth and IgM-PFC development in frequencies (Table 2) expected from polyclonal activation of a large part of all B cells (see Table 1). Both B blasts and small B cells were polyclonally activated. B-cell blasts stimulated by antigen-activated T-cell help (T'_{HRBC} + HRBC) responded polyclonally also in the presence of a second, unrelated antigen (SRBC). HRBC-, SRBC- and TNP-SRBC specific B-cell clones grew and matured in frequencies comparable to those found with LPS-stimulation (20). Without mitogen or when the helper T cells plus adherent cells were exposed to no or an unrelated (SRBC) antigen, the B blasts did not continue to grow. This, once again, points to the antigen specificity of the helper T cells and the requirement for specific antigen in the production of nonspecific "help" (4, 9).

Antigen-activated T-cell help, however, stimulated specifically, and apparently exclusively, the fraction of small B cells for which the antigen was present in the cultures. These antigen-specific B-cell clones grew in frequencies comparable to those obtained by polyclonal LPS activation (14, 20). A second antigen (SRBC), added as a "bystander," led to the activation

Table 1. Total stimulation of mitogen-activated B-cell blasts* and of resting small B cells† by LPS or by antigen-activated T-cell help, measured by thymidine uptake and the development of IgM-secreting PFC

Measurement	Mode of stimulation: LPS	T'_{HRBC} + HRBC‡	None
Thymidine uptake, ^{3}H cpm per 10^5 cultured cells			
B blasts	62,000 ± 8000	55,000 ± 6000	7000 ± 2000
Small B cells	42,000 ± 5000	200 ± 100	150 ± 100
Frequency of reactive cells			
B blasts	1 in 1–3	1 in 1–3	<1 in 300
Small B cells	1 in 3–10	<1 in 100	<1 in 300
IgM-secreting PFC (average of 10 cultures)§			
Plated B blasts			
300	3500 ± 820	850 ± 530	80 ± 10
100	1200 ± 510	500 ± 350	30 ± 5
30	450 ± 380	210 ± 200	12 ± 2
10	125 ± 110	64 ± 55	5 ± 2
3	15 (20, 32, 18, 0, 0, 15, 24, 18, 18, 8)	13 (10, 18, 12, 0, 15, 0, 22, 17, 31, 15)	0
0	0	0	0
Plated small B cells			
300	1050 ± 450	150 ± 120	<5
100	300 ± 220	35 ± 10	<5
30	75 ± 62	10 ± 5	<5
10	25 (45, 15, 22, 0, 36, 41, 34, 2, 47, 15)	<5	<5
3	10 (0, 0, 31, 0, 25, 0, 2, 3, 41, 0)	<5	<5
1	4	<5	<5
0	<5	<5	<5

± indicates SD.

* Generated in a 2-day activation period with LPS (50 μg/ml), purified from nonactivated small cells by velocity sedimentaion. C57BL/6J *nu/nu* spleen.

† Purified from medium- and large-sized cells of spleen by velocity sedimentation. C57BL/6J *nu/nu* spleen.

‡ Helper T cells of C57BL/6J origin specific for HRBC, kept as continuous line, in the presence of C57BL/6J adherent cells and antigen (HRBC), irradiated with 3000 rads.

§ All cultures yielded at least five IgM-PFC except those in which the limiting dilution of growth-inducible B cells led to no precursors in some cultures and consequently to responses fluctuating in the 10 individual cultures. Wherever such fluctuations were observed, the raw data of the 10 individual cultures are given in parentheses.

Table 2. Antigen-specific stimulation of LPS-activated B-cell blasts* and of resting small B cells†

Specificity of reactive B cell	Frequencies of specific B cells after restimulation with: LPS	T'_{HRBC} + HRC‡	T'_{HRBC} + HRBC + SRBC§	None, T'_{HRBC} + SRBC, or T'_{HRBC} alone¶
	LPS-activated B-cell blasts			
Anti-HRBC	1 in 1000–3000	1 in 1000–3000	1 in 1000–3000	<1 in 300,000
Anti-SRBC	1 in 3000–10,000	1 in 10,000–30,000	1 in 10,000	<1 in 300,000
Anti-TNP-SRBC	1 in 100–300	1 in 300–1000	1 in 300	<1 in 300,000
	Resting small B cells			
Anti-HRBC	1 in 3000–10,000	1 in 3000–10,000	1 in 3000–10,000	<1 in 300,000
Anti-SRBC	1 in 10,000	<1 in 300,000	1 in 10,000	<1 in 300,000
Anti-TNP-SRBC	1 in 300–1000	<1 in 300,000	<1 in 300,000	<1 in 300,000

*, †, and ‡: see footnotes *, † and ‡ in Table 1.

§ HRBC-specific helper T cells, adherent cells (both irradiated), and specific (HRBC) as well as "bystander" (SRBC) antigen.

¶ HRBC-specific help and wrong (SRBC) antigen gave the same results as no antigen added to T'_{HRBC} or no helper cells, macrophages, or antigen added at all.

of SRBC-specific clones (4) in frequencies comparable to those measured by LPS activation. Clones specific for a third antigen (TNP-SRBC), not present in the cultures, were not induced to grow. (The latter data indicate that coupling of TNP groups to SRBC apparently masks most of the antigen normally detected on unsubstituted SRBC.)

Taken together, Tables 1 and 2 suggest that activation of division and maturation of resting small B cells by antigen-activated T-cell help is antigen-dependent and specific.

H-2-unrestricted polyclonal activation of B-cell blasts and H-2-restricted antigen-specific activation of small B cells

LPS-activated B-cell blasts and small B cells were prepared from a number of mouse strains totally or partly allogeneic to the antigen-activated T-cell help [helper T cells and adherent cells both of C57BL/6J (*b b b*) origin]. LPS-activated B blasts of either strain could be restimulated polyclonally, just as with LPS (Table 3), confirming earlier results (10). Approximately one in 1000–3000 IgM-PFC were specific for the antigen (HRBC) present in the cultures, as found previously for LPS-activated B cells (20).

LPS stimulation of resting small B cells yielded peak IgM-PFC responses on day 5 (Fig. 1) with all strains of mice and in numbers (Table 3) expected of cell populations in which 10–30% of all B cells are LPS reactive (see also Table 1). With small B cells of all strains, antigen-activated T-cell help induced IgM-PFC responses 1/20th to 1/50th the magnitude of those obtained by LPS stimulation (Table 3, Fig. 1). As already indicated (see Table 1), the majority of these IgM-PFC developed by maturation without division.

About one in 1000–3000 IgM-PFC developed by LPS stimulation of small B cells were HRBC-specific (Table 3), coinciding with previous frequency determinations (20). High HRBC-specific PFC responses were obtained with C57BL/6J *nu/nu* and with B10.A(5R) small B cells. The anti-HRBC-specific response at day 5 was, in fact, approximately 10-fold above that measured after LPS activation, even though the frequency of HRBC-specific B-cell clones was the same (Table 2). The HRBC-specific B-cells continued to grow here up to day 7, in contrast to the LPS-stimulated cells (Fig. 1). The likely reason is that LPS stimulates many small cells to divide and mature, whereas antigen-activated T-cell help stimulates only the much less frequent specific B-cell clones, exhausting nutrients in the medium more slowly. No HRBC-specific PFC above the background could be detected with small B cells of C3H/Tif *nu/nu*, BALB/c *nu/nu*, or B10.A(4R) mice. Maturation of IgM-PFC without growth (Table 1) stimulated in an H-2-unrestricted fashion (Table 3) is polyclonal with a peak at day 5 (Fig. 1). Therefore, HRBC-specific IgM-PFC, which are one in 1000–3000 of all IgM-PFC, were not detectable above background because only 50–500 total IgM-PFC developed in these cultures.

These data suggest that the antigen-specific activation of resting small B-cells to divide and secrete specific antibody is H-2 restricted. The restriction is mapped to the *K* end of the *H-2* complex.

Histoincompatible small B cells (C3H/Tif) failed to show an HRBC-specific response even when compatible F_1 adherent cells (C3H/Tif × C57BL/6J) were present in the cultures (Table 3). This result points to helper T-cell–B-cell rather than adherent-cell– B-cell interactions (9).

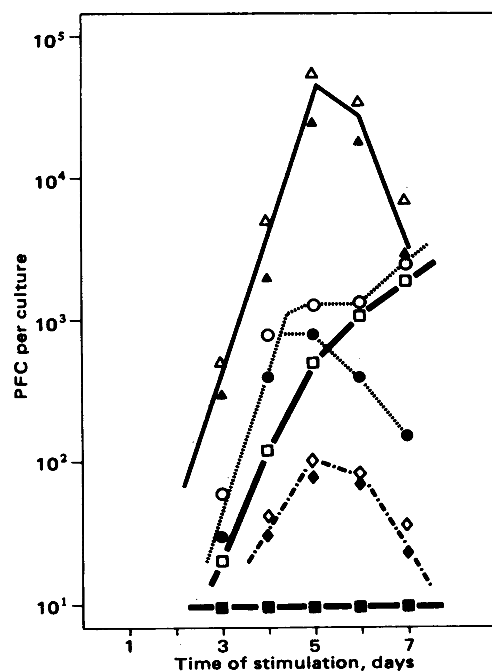

FIG. 1. Activation of C57BL/6J *nu/nu* (open symbols) and of C3H/Tif *nu/nu* (closed symbols) resting small spleen cells by LPS (△, ▲, ◇, ◆) or by antigen-activated T-cell help (T'_{HRBC} and HRBC) (○, ●, □, ■) as measured by the development of total IgM-secreting PFC (△, ▲, ○, ●) or of antigen (HRBC)-specific, direct (IgM) PFC (□, ■, ◇, ◆).

Table 3. H-2 restriction of the antigen-specific response of mitogen-activated B-cell blasts* and of resting small B-cells† to stimulation by LPS and by antigen-activated T-cell help

					LPS-activated B-cell blasts stimulated by:				Resting small B cells stimulated by:			
					LPS		T_{HRBC} + HRBC‡		LPS		T_{HRBC} + HRBC‡	
B cells¶	H-2 haplotype *K*	*I-A*	*D*	Response§	Anti-HRBC PFC	Total IgM-PFC	Anti-HRBC PFC	Total IgM-PFC	Anti-HRBC PFC	Total IgM-PFC	Anti-HRBC PFC	Total IgM-PFC
Mouse strains												
C57BL/6J												
nu/nu	*b*	*b*	*b*		85	35,000	25	10,000	100	55,000	500	1250
C3H/Tif *nu/nu*	*k*	*k*	*k*		70	35,000	15	8,000	80	25,000	<5	800
BALB/c *nu/nu*	*d*	*d*	*d*		50	25,000	30	10,000	85	35,000	<5	750
B10.A(4R)	*k*	*k*	*b*		65	45,000	35	12,000	70	50,000	<5	1000
B10.A(5R)	*b*	*b*	*d*		70	35,000	25	11,000	75	65,000	650	950
Chimeric mice‖												
CBA/J →												
CBA/J × C57BL/6J												
k k k → *k k k* × *b b b*					10	6,000	12	7,000	100	45,000	<5	950
With CBA/J × C57BL/6J F_1 (*k k k* × *b b b*) adherent cells "*in vitro*"												
C57BL/6J												
nu/nu	*b*	*b*	*b*		75	30,000	30	12,000	85	40,000	700	1000
C3H/Tif *nu/nu*	*k*	*k*	*k*		60	30,000	25	15,000	95	35,000	<5	800

*, †, and ‡: see footnotes *, †, and ‡ of Table 1.

§ Measured at day 5 of stimulation of small resting cells (5 × 10^4 per culture) and at day 3 of restimulation of blasts (1 × 10^4 per culture) (see also Fig. 1). Direct IgM-PFC were measured in the case of anti-HRBC-PFC.

¶ B10.A(4R), B10.A(5R), normal small spleen cells, CBA → CBA × C57BL/6J F_1 chimeric small spleen cells, and CBA/J × C57BL/6J F_1 adherent cells of spleen were treated with anti-Thy-1.2 and complement.

‖ Instead of LPS-activated B-cell blasts, "background" activated B-cell blasts of spleen, separated by velocity sedimentation, were used as source of activated cells.

DISCUSSION

In vivo experiments by Sprent (6, 7) show that successful T-cell–B-cell collaboration is H-2 restricted and confined to Ia antigens on B cells that the helper T-cells have encountered in the thymus during their early differentiation. Our *in vitro* data are in line with these findings and disagree with others showing no H-2 restriction for T-cell–B-cell collaboration (21, 22). Such a discrepancy could arise for at least three reasons.

First, we have distinguished the reactions of small B cells and B blasts to antigen-activated T-cell help. Disagreement between *in vitro* experimental results may well be due to this difference in action of T-cell help on resting and on activated B cells. B-cell blasts are stimulated by T-cell help in an H-2-unrestricted fashion (10) and these blasts, forming part of all normal lymphocyte preparations, may well be the prime targets of stimulation by T-cell help in experiments *in vitro*, giving the impression that T–B collaboration is H-2 unrestricted (21, 22). Antigen-activated T-cell help causes polyclonal maturation, but without replication, of small B cells to IgM-PFC. This stimulation is also H-2 unrestricted. Some of the apparent H-2-unrestricted effects observed by others could therefore also be simple maturation without clonal growth.

Second, our antigen-specific helper T cells are free from possible contamination by alloreactive helper T cells, suppressor T cells, or helper T cells of other specificities, due to long-term culturing and cloning (4).

Third, improved culture conditions for murine lymphocytes have allowed increased survival of lymphocytes in culture. By replacement of serum with albumin, transferrin, and lipids (13, 17) we avoid stimulatory or suppressive factors that are often present in serum.

From our findings, the following picture of T–B collaboration may be drawn.

Initially, all cells of the immune system are expected to be in their resting state. Cell biology defines this state as G_0, in which neither DNA synthesis nor mitosis occurs. The mitotic cycle starts with DNA synthesis (S-phase), followed by a gap (G_2) before division (M) occurs. Many types of cells, including B cells, have been shown to require the growth-stimulating hormone (or factor, or mitogen) soon after M and early in the gap (G_1) preceding S phase. For example, activated B-cell blasts will not continue to divide (but will continue to secrete Ig) when the stimulatory mitogen is removed, and will resume the cycle when either the same stimulatory substance or another growth factor is added for which this B cell possesses reactivity and hence probably receptors (14). It appears also that growth-stimulating factors for a number of cell types, including B cells, are used up and must therefore be constantly supplied to keep the cells dividing. The cellular source of these growth factors may be either the antigen-specific helper T cell or the macrophages that interact with the helper T cell. We imply that one factor (F_B) induces both maturation *and* replication, although it is equally possible that separate factors govern these two reactions of a B cell.

For a resting G_0 B cell to respond to F_B by division, it has to be excited. The excited state is the condition of a B cell in which the interaction with F_B leads to mitosis. Excitation is not brought about by single occupancy of either Ig (through antigen) or Ia (through Ia-complementary structures of T-cell help) alone, but only by *dual* occupancy of both.

The role of Ig and Ia in dual recognition leading to "excitation" of a resting B cell remains to be elucidated. The observed activation of specific resting B cells by "bystander" antigen (23) (see Table 2) suggests that antigen and I complementarity of T-cell help can be presented separately to the resting B cell. In principle this should allow the activation of specific B-cell clones for any antigen "*in vitro*" as a bystander of a first antigen–helper T-cell–macrophage interaction. An excited B cell is

viewed as an activated B-cell blast in the G_1 phase of the cell cycle. It will enter the cell cycle and go through S, G_2, and M only if F_B is provided. Neither antigen nor I complementarity, even acting together, is able to stimulate the first or next round of division. In fact, our experiments suggest that neither is needed for stimulation by F_B once the resting B cell has become excited, because B-cell blasts respond polyclonally and in an H-2-unrestricted fashion. In summary, we distinguish excitation of a resting B cell from proliferation of an excited B cell. Immune induction of a B cell thus involves three control elements: Ig, Ia, and B growth factor receptors.

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