EVIDENCE THAT Lyb-2 IS CRITICAL TO SPECIFIC
ACTIVATION OF B CELLS BEFORE THEY BECOME
RESPONSIVE TO T CELL AND OTHER SIGNALS*

BY HIDETAKA YAKURA, FUNG-WIN SHEN, ELAINE BOURCET, AND
EDWARD A. BOYSE‡

From the Memorial Sloan-Kettering Cancer Center, New York 10021

That part of B cell differentiation which begins in the reaction of antigen with
surface immunoglobulin (sIg) receptors and ends in the production of antibody-
secreting cells comprises a complex train of discrete events (1, 2). An important aspect
of this process concerns the chemical signals, positive and negative, and their corre-
sponding receptors, whereby the steps in this process are regulated. In this report, we
present further evidence for the participation of Lyb-2, a cell surface component
expressed only in the B cell lineage, in the mechanism of B cell activation.

We have already shown that monoclonal Lyb-2 alloantibody (mc-α-Lyb-2) inhibits
the generation of antibody-forming cells (AFC) to sheep erythrocytes (SRBC) but not
to the T-independent antigens trinitrophenylated (TNP)-Ficoll and TNP-Brucella
abortus. This inhibition cannot be ascribed to a change in the kinetics of AFC
generation nor to any known variety of suppression (3). Because mc-α-Lyb-2.1 did
not inhibit generation of AFC from Lyb-2.1/Lyb-2.2 heterozygous cells, we inferred
that Lyb-2 antibody causes inhibition by blocking rather than by providing a negative
or suppressive signal (3).

To define the function of Lyb-2 more closely, we have now tested the ability of mc-
α-Lyb-2.1 to block the ability of B cells to receive signals that are known to be
required for differentiation of B cells to AFC. These data signify that Lyb-2 is not
concerned in the reception of soluble factors contained in mixed lymphocyte culture
(MLC) or macrophage (Mφ) culture supernatants (SN), but is involved in an early
phase of activation before B cells become overtly receptive to such differentiative
signals.

Materials and Methods

Mice. Except for C57BL/6 mice (purchased from The Jackson Laboratory, Bar Harbor,
ME), all were obtained from colonies at Memorial Sloan-Kettering Cancer Center.

Antigens. SRBC and horse erythrocytes (HRBC) were purchased from Gibco Diagnostics
Laboratories, Madison, WI, and Colorado Serum Co., Denver, CO, respectively.

Antibody. Monoclonal α-Lyb-2.1 antibody (clone 9-6.1) is described elsewhere (3).

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‡ American Cancer Society research professor of cell surface immunogenetics.

Abbreviations used in this paper: α, anti; AFC, antibody-forming cells; C, complement; FBS, fetal bovine
serum; HRBC, horse erythrocytes; IL-1, IL-2, interleukin 1, 2; LPS, lipopolysaccharide; mc, monoclonal;
MDM, Mishell-Dutton medium; Mφ, macrophages; MLC, mixed lymphocyte culture; PFC, plaque-
forming cells; sIg, surface immunoglobulin; SN, supernatant; SRBC, sheep erythrocytes; TCGF, T cell
growth factor; TNP, trinitrophenylated.
Cell Preparations

T-DEPLETED SPLEEN CELLS. T cells were eliminated by exposure of spleen cells (3 × 10⁷/ml) to mc-α-Thy-1.2 (1:100) and then, after washing, to selected rabbit serum (complement [C] 1:9). The cycle was then repeated with mc-α-Lyt-1.2 (1:25) plus mc-α-Lyt-2.2 (1:25) in place of α-Thy-1.2. The two treatments were needed because spleen cells subjected to only the first treatment did generate substantial numbers of α-SRBC PFC when exposed to mitogenic quantities of concanavalin A (4 µg/ml), whereas twice-treated cells did not. The fact that the two treatments reduced α-SRBC PFC generation by 95% in the presence of purified T cell growth factor (TCGF; kindly provided by Dr. Steven Gillis, Fred Hutchinson Cancer Research Center, Seattle, WA) further emphasizes the efficacy of the dual procedure in eliminating residual T cells.

B CELLS. To remove Mφ, spleen cells (~18 × 10⁷) were passed over a Sephadex G-10 column (bed volume, 30 ml; Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) and eluted with ~30 ml of RPMI 1640 (Grand Island Biological Co. Grand Island, NY) containing 20% fetal bovine serum (FBS) streptomycin (100 µg/ml) and penicillin (100 U/ml) (4). Recovery was consistently 50-60% of the starting population. This treatment did not change the proportion of T and B cells significantly, but reduced the esterase-positive cells from 5-9% to <0.5% (5). Efficacy of this procedure was further confirmed by functional assays (see Results). The eluted cells were treated with mc-α-Thy-l.2 plus mc-α-Lyt-1.2 and mc-α-Lyt-2.2, and C, to eliminate T cells (see above).

Mφ. Spleen cells (5 × 10⁷) in 15 ml of RPMI 1640 plus 20% FBS were plated in each 100-mm plastic culture dish and incubated at 37°C in a humidified atmosphere of 10% CO₂ and 90% air for 2 h. Nonadherent cells were then washed away, and adherent cells detached with a rubber policeman. The adherent cells were treated with mc-α-Thy-1.2 plus C and irradiated (1,500 rad). In the following text, these adherent Thy-1⁺ and radioresistant cells are referred to as Mφ.

Mishell-Dutton Culture. Spleen cells, T depleted or purified (as above), were cultured in RPMI 1640 supplemented with 10% FBS, L-glutamine (2 mM), 2-mercaptoethanol (5 × 10⁻⁵ M), sodium pyruvate (1 mM), streptomycin (100 µg/ml), penicillin (100 U/ml), and Hepes buffer (10 mM) in 16-mm flat-bottomed Linbro plates (Linbro Chemical Co., Hamden, CT) with SRBC (5 × 10⁶) at 37°C in a humidified atmosphere of 10% CO₂ and 90% air with daily feeding as described elsewhere (3).

Plaque-forming Cell (PFC) Assay. Direct α-SRBC or α-HRBC PFC counts were determined on day 5 by the slide version (6) of the Jerne plaque assay. Results are expressed as mean PFC counts per culture ± SEM or a percentage of the standard control PFC response (100 [PFC experiment]/[PFC control]).

Preparation of Factors

MLC-SN. 5 × 10⁶ B6 spleen cells were stimulated with 5 × 10⁶ irradiated DBA/2 spleen cells (2,000 rad) in 1 ml of Mishell-Dutton medium (MDM) in a Linbro well. 24 h later, the supernatant was centrifuged, passed through 0.45-µm filter (Millipore Corp., Bedford, MA), and stored at −20°C. This preparation showed a low but significant level of interleukin 2 (IL-2) activity determined by TCGF assay, and may contain a factor similar to late-acting T cell-replacing factor. The B cell helper activity of Mφ-derived factors (e.g., IL-1) was minimized by reducing the concentration of MLC-SN (see Results).

SUPERNATANT FROM P388D.1 CELLS (MF-SN). As a source of Mφ-derived factors, 2 × 10⁶ P388D.1 cells (a DBA/2 Mφ cell line kindly provided by Dr. Peter Ralph, Memorial Sloan-Kettering Cancer Center) were cultured in 1 ml of MDM with 0.01 µg/ml Escherichia coli lipopolysaccharide (LPS; 055:B5, Difco Laboratories, Detroit, MI) in a Linbro well for 4 d (7). The cell-free SN was dialyzed against 100 vol of RPMI 1640 for 24 h, filtrated, stored at −20°C, and used at a concentration of 0.5%.

Results

Properties of the MLC-SN and MF-SN Preparations Used, and Relative Homogeneity of the Selected Cell Population Tested (Data in Table I). Without MLC-SN, no PFC were
Properties of the MLC-SN and Mφ-SN Preparations Used, and Relative Homogeneity of the Selected Cell Populations Tested

| Soluble factors | Spleen cells |
|-----------------|--------------|
| Concentration of MLC-SN (added on day 2) | Mφ-SN (0.5%) | B + Mφ (T depleated: 3 x 10^6) | B (T) and Mφ depleted: 5 x 10^5 | Mφ (× 10^-4) | α-SRBC PFC per culture |
| % | | | | | |
| First experiment | | | | | |
| 30 | + | - | - | 8605 ± 429 |
|  | - | + | - | 3924 ± 331 (54 %§) |
| 20 | + | - | - | 4185 ± 94 |
|  | - | + | - | 798 ± 27 (81 %§) |
|  | - | - | + | 2 | 1401 ± 85 |
|  | - | + | 4 | 3822 ± 370 |
|  | - | - | 8 | 4141 ± 254 |
|  | - | 8 | 0 ± 0 |
| 10 | + | - | - | 1800 ± 102 |
| none | + | - | - | 96 ± 13 (95 %§) |
|  | - | + | - | 0 ± 0 |
|  | - | - | 8 | 0 ± 0 |
| Second experiment | | | | | |
| 15 | - | - | + | 479 ± 19 |
| + (day 0) | - | + | - | 4070 ± 461 |
| + (day 1) | - | + | - | 330 ± 20 |
|  | + (day 0) | - | + | - | 160 ± 10 |
| none | + (day 1) | - | + | - | 0 ± 0 |

* Two of several similar experiments are shown.
† See Materials and Methods.
§ Percent reduction caused by removal of Mφ = 100 (1 - a/b), where a = response for B cells alone, and b = response for B + Mφ.

Evidence That Lyb-2 Antibody Acts on B Cells and Not on Mφ (Table II).

Thus, the Mφ depletion procedure is evidently adequate. At high concentration, the MLC-SN preparation used has appreciable Mφ-replacing activity (possibly of Mφ origin, e.g., IL-1), but this activity is negligible at the selected concentration of 10-15% MLC-SN. Therefore, under the conditions described, the helper actions of MLC-SN and Mφ-SN can be ascribed to T cells and Mφ, respectively, and both are required for maximal PFC response.
Table II

Lyb-2 Antibody Acts on B Cells, Not on Mφ

| Composition of culture | α-SRBC per culture |
|-----------------------|--------------------|
|                       | With mc-α-Lyb-2.1 (A) | Without mc-α-Lyb-2.1 (B) | Percent response (100A/B) |
| B6-Lyb-2.1 (8)        | 226 ± 23            | 2288 ± 169               | 10         |
| B6-Lyb-2.1 (4)        | 432 ± 58            | 1636 ± 86                | 26         |
| B6                    | 2693 ± 113          | 3126 ± 276               | 86         |
| B6                    | 1283 ± 73           | 1649 ± 177               | 78         |
| B6-Lyb-2.1 (8)        | 745 ± 95            | 3064 ± 177               | 19         |
| B6-Lyb-2.1 (4)        | 452 ± 70            | 1356 ± 88                | 29         |
| B6                    | 2999 ± 110          | 2780 ± 216               | 108        |
| B6                    | 1516 ± 67           | 1397 ± 129               | 109        |

Combinations of B cells and Mφ, as indicated, were cultured with SRBC in the presence or absence of 2% mc-α-Lyb-2.1. On day 2, 15% MLC-SN was added to each culture, and α-SRBC PFC were assayed on day 5.

* PFC responses of B cells with neither Mφ nor MLC-SN were nil for B6-Lyb-2.1 and B6 cells. Responses of B cells with MLC-SN but without Mφ were 183 ± 15 for B6-Lyb-2.1 cells and 250 ± 30 for B6 cells.

† PFC responses of Mφ with and without MLC-SN were nil.

Lyb-2 Antibody Does Not Block Reception of Helper Factors Present in MLC-SN or Mφ-SN (Table III). The fact that Lyb-2 antibody blocked generation of PFC to T-dependent but not to T-independent antigen (3) could mean that Lyb-2 antibody interferes with a reaction between T and B cells, one such reaction being the reception of T helper factors by B cells. Accordingly, having shown that the background helper activity attributable to Mφ factors in 15% MLC-SN is negligible (Table I), we tested the possibility that Lyb-2 molecules serve as receptors for T helper factors. For this purpose, we used T-depleted spleen cell (B + Mφ) as responder cells, and MLC-SN (15%), added at 44 h, as a source of T helper factors. Representative data are given in Table III (experiment 1). The rationale for these experiments was that if Lyb-2 antibody acted simply by blocking the receptor sites for T helper factors, then Lyb-2 antibody added at 44 h, the time of adding MLC-SN, should effectively block the response. In the event, when antibody was added at the initiation of culture, there was maximal PFC reduction, but when antibody was added at 44 h there was no reduction. These results indicate that Lyb-2 is not involved in the reception of T helper factors present in MLC-SN, and imply that Lyb-2 functions at some point in a process that occupies about 44 h after stimulation by antigen and precedes the operation of T helper factors. This places the time of action of Lyb-2 antibody within

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Lyb-2 Antibody Does Not Block Reception of Helper Factors Present in MLC-SN or Mφ-SN

| Experiment | Responding cells (5 × 10⁶) | Time of addition of: | MLC-SN (15%) at 44 h | Cell donor: PFC response on day 5 |
|------------|-----------------------------|---------------------|----------------------|--------------------------------|
|            | mc-α-Lyb-2.1 (1:100) | Mφ-SN (0.5%) |                       | B6-Lyb-2.1 B6 (control) |
| 1* B + Mφ (T depleted) | — | + | 100 | 100 |
| — | — | — | 0 | 0 |
| 0 h | + | 13 | 92 |
| 20 h | + | 50 | 93 |
| 44 h | + | 113 | 101 |
| 0 h | 0 h | + | 24 | 90 |
| 20 h | 0 h | + | 40 | 107 |
| 44 h | 0 h | + | 91 | 121 |
| 0 h | 44 h | + | 17 | 95 |
| 20 h | 44 h | + | 55 | 98 |
| 44 h | 44 h | + | 89 | 110 |

* One of four similar experiments.
† Standard responses (without antibody) per culture: B6-Lyb-2.1, 5545 ± 974 (experiment 1), 3190 ± 442 (experiment 2), and 2634 ± 298 (experiment 3); and B6, 3980 ± 291 (experiment 1), 4070 ± 461 (experiment 2), and 2257 ± 80 (experiment 3).
§ One of two similar experiments.

Period of Action of Lyb-2 Antibody

| Period of culture with mc-α-Lyb-2.1 (1:100) plus SRBC | PFC response |
|--------------------------------------------------------|--------------|
|                                                        | % standard   |
| 2 h                                                     | 79           |
| 4 h                                                     | 72           |
| 20 h                                                    | 23           |

Purified B cells (5 × 10⁶) from B6-Lyb-2.1 mice were cultured with SRBC plus mc-α-Lyb-2.1 for the period of time indicated, after which SRBC were lysed and the cells washed four times. 5 × 10⁶ of these cells were then cultured with SRBC, with addition of 0.5% Mφ-SN at 20 h and 10% MLC-SN at 44 h.

* 5 d after exposure to SRBC: 100 (PFC with antibody)/(PFC without antibody).

the phase of B cell:Mφ interaction, and focuses attention on the reception of Mφ signals by B cells or on the actual triggering of B cells by antigen.

Experiments 2 and 3 of Table III were directed to the former possibility, namely that reception of Mφ signals is impeded by Lyb-2 antibody. Purified B cells were used
as responder cells, P388D.1 supernatant as a source of Mφ-derived factors (Mφ-SN), and MLC-SN as a source of T helper factors. The rationale was again that if Lyb-2 antibody blocks reaction of Mφ-derived factors with B cells, then antibody should inhibit the PFC response when added together with Mφ-SN at 0 or 44 h. This is not the case: when Mφ-SN and antibody were added together at the initiation of culture, there was maximal PFC reduction (experiment 2), but when Mφ-SN and antibody were added together at 44 h, there was very little reduction (experiment 3). These data imply that reception of Mφ-derived factors represented in P388D.1 Mφ-SN is not blocked by Lyb-2 antibody.

**Evidence That Lyb-2 Antibody Acts During the Triggering of B Cells by Antigen**

**Period of Action of Lyb-2 Antibody.** Purified B cells were exposed to SRBC plus Lyb-2 antibody for 2–20 h, freed of SRBC, washed, and cultured with fresh SRBC. Mφ-SN (0.5%) and MLC-SN (10%) were added at 20 and 44 h, respectively. Table IV indicates that maximal reduction of PFC by Lyb-2 antibody requires the presence of this antibody for >4 and <20 h after exposure to antigen.

**Only antigen-Triggered B Cells are Affected by Lyb-2 Antibody.** In the experiments summarized in Table V, purified B cells were cultured with Lyb-2 antibody for 20 h, with or without SRBC, and the SRBC and excess antibody then removed. The cells were then cultured again, without antibody, with either SRBC or HRBC. Mφ-SN and MLC-SN were added at 20 and 44 h, respectively, after the first exposure to SRBC or HRBC (whichever was to assayed for PFC response). Group A of Table V shows again (as in Table IV) that exposure to SRBC plus Lyb-2 antibody (1st culture) abolished the subsequent capacity of the responding population (2nd culture) to give a maximal PFC response to SRBC in the absence of Lyb-2 antibody. Group B shows that exposure to Lyb-2 antibody alone in the absence of antigen did not affect the subsequent response to SRBC. Group C shows that the exposure to SRBC plus Lyb-2 antibody did not affect the subsequent PFC response to HRBC. Thus, Lyb-2 antibody evidently acts during the triggering process induced by reaction of antigen with B cells. Blocking by Lyb-2 antibody activity cannot be explained simply as a negative effect on all B cells regardless of their specificities.

**Discussion**

Of the several possible mechanisms whereby Lyb-2 antibody might block T-assisted generation of PFC, some can now be excluded. Lyb-2 antibody does not interfere
with reception of Mφ-derived factors in the supernatant of P388D.1 cells, nor with the reception of MLC-associated factors, and its time of action is previous to the acquisition of responsiveness to these signals. This places the function of Lyb-2 within the period of antigen-triggered B cell stimulation. The fact that Lyb-2 antibody blocks the generation only of PFC derived from B cells concurrently exposed to antigen (the remaining B cells responding normally to a subsequent second antigen after Lyb-2 antibody is removed) indicates that the Lyb-2 cell surface component is somehow involved in a differentiative process ('proliferative' step) initiated by binding of antigen. The Lyb-2 molecule is not the slg receptor for antigen: its structure is inappropriate (8), rabbit α-mouse Ig does not block serological absorption of α-Lyb-2 (unpublished data), and there is no known reason why blocking of the antigen receptor should affect only T-dependent antigen.

Two sorts of action, not necessarily exclusive of one another, can be suggested for the function of Lyb-2. First, Lyb-2 may be involved in internal transmission of a proliferative stimulus, for example, the mitogenic component in FBS, which in our experience (unpublished results) is needed for an α-SRBC response but is not essential to demonstrable TNP-Ficoll and TNP-Brucella responses. In the same context, Lyb-2 may participate in molecular rearrangements ('cross-linking') of slg receptors required for response to T-dependent antigen.

Second, Lyb-2 molecules might be concerned in the phenotypic expression or presentation of receptors for differentiative signals from Mφ or T cells, presumably affecting their numbers, affinity, or conformation. The finding that MLC-SN is more effective on day 2 of culture than at the start (9) may be a hint that the receptor phenotype, as well as the number of responsive B cells, is important to efficient PFC generation. In this context, the report that resting or unstimulated B cells were not responsive to proliferative and differentiative signals until stimulated by LPS (10) makes us wonder whether the expression and display of signal receptors on B cells are subject to the same kinds of regulation as hormone receptors in classical endocrine system (11, 12).

Summary

The generation of plaque-forming cells (PFC) to T-dependent antigen, but not to T-independent antigen, is reduced in vitro by Lyb-2 antibody. Monoclonal Lyb-2 antibody, added to Mishell-Dutton cultures within the first 2 d, but not later, greatly reduces the generation of α-sheep erythrocyte (SRBC) PFC from T-depleted spleen cells whether help is provided in the form of intact T cells or as soluble factors contained in mixed lymphocyte culture (MLC) supernatants. Generation of α-SRBC PFC from purified B cells, assisted by soluble factors in MLC and macrophage (P388D.1 cell) supernatants, is similarly reduced by Lyb-2 antibody. The initial 2-d period, during which cultures are diminishingly sensitive to reduction of PFC generation by Lyb-2 antibody, is not affected by the time at which such soluble factors are added. Thus, Lyb-2 cell surface molecules evidently do not function as receptors for these differentiative signals.

Reduction of PFC generation by Lyb-2 antibody is antigen dependent in the sense that reduction of the PFC response to one antigen (SRBC) does not affect subsequent generation of PFC to a second antigen (horse erythrocytes) from the same cell population. These findings accord with the view that the Lyb-2 molecule participates...
in a B cell differentiative phase, probably proliferative, which begins with binding of antigen and precedes the phase in which B cells become fully receptive to signals from T and other cells.

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