Lyb-2 SYSTEM OF MOUSE B CELLS
Evidence for a Role in the Generation of
Antibody-forming Cells*

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The cell-surface component identified by the Lyb-2 system of alloantigens (1-5) is
confined to B cells (1, 4). Antibody-secreting B cells, however, are Lyb-2- (6). Thus if
the exclusive expression of Lyb-2 on B cells implies a relation to B cell function, then
Lyb-2 presumably participates in the generative phase of the antibody response.
Evidence to that effect is given in this report.

Materials and Methods

Mice. Mice 6-10 wk of age were obtained from colonies maintained by E. A. Boyse at
Memorial Sloan-Kettering Cancer Center, New York, and were matched for sex and age in
each experiment.

Antigens. Sheep erythrocytes (SRBC) were purchased from Colorado Serum Co., Denver,
Colo. and GIBCO Diagnostics, Madison, Wis. Trinitrophenyl (TNP)-Ficoll (TNPg-FicolI) and
TNP-Brucella abortus were kindly provided by Dr. James Mond and Dr. William Paul of the
National Institutes of Health, Bethesda, Md.

Monoclonal Lyb-2.1 Antibody. For the production of monoclonal Lyb-2.1 antibody (mc-α-Lyb-
2.1), spleen cells from BALB/c mice (Lyb-2.2) immunized with DBA/2 spleen cells (Lyb-2.1)
were fused with P3/NSI/1-Ag4-1 according to the method of Kohler and Milstein (7), yielding
a clone (9-6.1) producing antibody against Lyb-2.1. Specificity for Lyb-2.1 was established by
strain distribution, including the critical distinction of B6 (Lyb-2.2) from the congenic B6-Lyb-
2.1 strain which was derived by typing with conventional Lyb-2.1 antiserum. Absorption
analysis, indicative of restriction to B cells, also conformed to findings with conventional Lyb-
2.1 antiserum (1). The titer of mc-α-Lyb-2.1, from ascites of BALB/c mice bearing 9-6.1 cells,
was 1:20,000 in cytotoxicity assay with Lyb-2.1 spleen cells, and the maximal proportion of
lysed spleen cells was 55-60%.

Immunoselection

Elimination of Lyb-2+ Cells. Equal volumes of a suspension of spleen cells were centri-
fuged and resuspended (3 × 107/ml) in mc-α-Lyb-2.1 (1:30) (part 1) or normal mouse serum
(NMS) (1:40, control) (part 2) for 30 min on ice; the cells were then spun down and resuspended
in pre-selected rabbit serum (source of complement [C], 1:7) for 30 min at 37°C. The cycle was
then repeated, and the cells washed twice. The concentration of the NMS-control cells was
then adjusted to 1 × 107/ml in Mishell-Dutton medium (MDM), and the Lyb-2-depleted cells
were suspended in the same volume of MDM. Counts of viable cells (trypan blue exclusion)

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Abbreviations used in this paper: AFC, antibody-forming cell(s); C, complement; FBS, fetal bovine serum;
mc-α-Lyb-2.1, monoclonal Lyb-2-1 antibody; mc-α-Thy-1.2, monoclonal Thy-1.2 antibody; MDM, Mishell-Dutton Medium; NMS, normal mouse serum; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TNP, trinitrophenylated.

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were recorded for both suspensions, the deficiency caused by Lyb-2 depletion was 55–60%, in accord with published data (1, 4).

Elimination of Lyt-2+ and Thy-1+ Cells. Lyt-2+ cells were eliminated from nylon-purified splenic T cells by one cycle of treatment as above, with a standard conventional Lyt-2.2 antiserum plus C (1:9) and cell counts were adjusted as above. The same procedure was used when eliminating Thy-1+ cells with monoclonal Thy-1.2 antibody (mc-a-Thy-1.2) (1:1,000).

Mishell-Dutton Culture. Spleen cells (1 × 10^7/ml) were cultured in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal bovine serum (FBS) (Microbiological Associates, Walkersville, Md.), l-glutamine (2 mM), streptomycin (100 μg/ml), penicillin (100 U/ml), 2-mercaptoethanol (5 × 10^-4 M), sodium pyruvate (1 mM), and Hepes buffer (10 mM), in 16-mm flat-bottomed plates (76-033-65; Linbro Chemical Co., Hamden, Conn.) with SRBC (5 × 10^6), TNP-B. abortus (1:1,000 of stock) or TNP-Ficoll (1 ng/ml), with or without mc-a-Lyb-2.1 (1:50 unless otherwise stated), at 37°C in a humidified atmosphere of 10% CO2 without rocking. Cultures were fed daily with a mixture of 60 μl nutritional cocktail (8) and 30 μl FBS.

Plaque-forming Cell (PFC) Assay. PFC counts were determined on day 5 for anti-SRBC (unless otherwise stated), and on day 4 for anti-TNP response to T-independent antigen, respectively, by the slide modification (9) of the Jerne plaque assay. TNP was coupled to SRBC as described (10). Results were expressed as PFC per culture or as the percent PFC response: \( \% \text{PFC response} = \frac{\text{PFC experiment}}{\text{PFC control}} \times 100 \).

Results and Discussion

Inhibition of Generation of Anti-SRBC PFC by Lyb-2 Antibody. Fig. 1 shows the PFC responses of cultures in which mc-a-Lyb-2.1 was included throughout the 5-d culture period. PFC counts were reduced in proportion to the concentration of Lyb-2.1 antibody, the greatest reduction, 73%, occurring at the highest concentration of mc-a-Lyb-2.1 tested, 1:50. Specificity for Lyb-2 is shown by the controls in which Lyb-2.1 antibody caused no reduction in PFC counts when B6 spleen cells (Lyb-2.2) were substituted for congenic B6-Lyb-2.1 spleen cells (Fig. 1).

Rate of PFC Generation in the Presence of Lyb-2 Antibody. Might the lower PFC counts at day 5 (Fig. 1) be a result of a change in rate of PFC generation, the response having peaked earlier or later than day 5 in the presence of Lyb-2 antibody?

Fig. 2 shows that this is not so. In the range of 1–7 d, PFC counts were maximal at
FIG. 2. Rate of anti-SRBC PFC generation in the presence of Lyb-2 antibody. Spleen cells from B6-Lyb-2.1 mice were cultured with \(5 \times 10^6\) SRBC in the presence (○) or absence (□) of mc-a-Lyb-2.1 (1:50). PFC were assayed on days 1–7. The response of control B6 cells (Lyb-2.2) was unaffected by the presence of mc-a-Lyb-2.1 (data not shown).

FIG. 3. Effects of Lyb-2 antibody added at increasing intervals after initiation of culture. mc-a-Lyb-2.1 (1:50) was added on days 1–4. Data expressed as in Fig. 1.

day 5 in the presence and absence of mc-a-Lyb-2.1, implying that Lyb-2 antibody does not alter the rate of generation of antibody-forming cells (AFC).

Period of Action of Lyb-2 Antibody. Fig. 3 shows that mc-a-Lyb-2.1 was about one-half as effective in reducing PFC counts when introduced after 24 h of culture, as compared with the first 12 h, and was ineffective after 3 d.

Non-T Identity of Cells Affected by Lyb-2 Antibody. Table I is concerned with the possibility that the cells on which Lyb-2 antibody acts, in lowering PFC generation, are not in fact B cells, but other cells whose Lyb-2 phenotype has previously escaped serological detection. This question was approached first with cultures composed of B6 or B6-Lyb-2.1 splenic T cells (nylon purified) combined with B6-Lyb-2.1 or B6 T-depleted spleen cells (mc-a-Thy-1.2 + C). Table I shows that PFC counts were reduced by mc-a-Lyb-2.1 only when T-depleted spleen cells were Lyb-2.1, the Lyb-2 phenotype of the T cells being irrelevant. Thus the cells on which Lyb-2 antibody acts are not T cells.

Evidence against the Involvement of Suppression. The data above suggest that B cell precursors of AFC are inhibited by Lyb-2 antibody. Alternatively, Lyb-2 antibody might cause B cells to evoke suppression from T cells. But the data in Table II are
### Table I

**Non-T Identity of Cells Affected by Lyb-2 Antibody**

| Composition of culture | Anti-SRBC PFC per culture | Percent response (100 A/B) |
|------------------------|----------------------------|---------------------------|
|                        |                            |                           |
| T-depleted spleen cells (8 x 10⁶) |                            |                           |
| Nylon-purified splenic T cells |                            |                           |
| B6-Lyb-2.1             | B6 (2 x 10⁶)*              | 466 ± 33                  |
| B6-Lyb-2.1             | B6 (1 x 10⁶)               | 406 ± 23                  |
| B6-Lyb-2.1             | —                          | <50                       |
| B6                    | B6-Lyb-2.1 (2 x 10⁶)       | 1,855 ± 167               |
| B6                    | B6-Lyb-2.1 (1 x 10⁶)       | 1,257 ± 134               |
| B6                    | —                          | <30                       |
| —                     | B6-Lyb-2.1 (2 x 10⁶)       | 1,855 ± 167               |

* Number of cells is in parenthesis.

### Table II

**Generation of PFC in Cultures Deprived of Lyt-2⁺ Cells**

| T cells added* | Anti-SRBC PFC per culture | Percent response (100 A/B) |
|----------------|----------------------------|---------------------------|
|                | With mc-α-Lyb-2.1          |                           |
|                | Without mc-α-Lyb-2.1       |                           |
| Unselected     | (A)                        |                           |
|                | (B)                        |                           |
| 2 x 10⁶        | 226 ± 89                   | 2,494 ± 330               |
| 1 x 10⁶        | 858 ± 82                   | 3,571 ± 163               |
| Lyt 1          | (2 x 10⁶‡)                 | 3,804 ± 584               |
|                | (1 x 10⁶‡)                 | 585 ± 153                 |

* B6-Lyb-2.1 nylon-purified T cells were added to 8 x 10⁶ T-depleted B6-Lyb-2.1 spleen cells. (Control PFC counts were 13 ± 2 for T cells alone and 140 ± 41 for T-depleted spleen cells alone.)

‡ Counts before selection with anti-Lyt-2.2 plus C. (Thus the numbers of Lyt1 cells in the unselected groups are the same as in the Lyt1 selected groups; see Materials and Methods.)

against such a mechanism, because elimination of Lyt-2⁺ cells (removing not only the Lyt23 T set to which T suppressors belong but also the antecedent Lyt123 set) did not abolish reduction of PFC by Lyb-2 antibody. Full reduction occurred with no T cells other than the Lyt1 set.

The data in Fig. 4 substantially eliminate the further possibility that Lyb-2 antibody evokes B cell-mediated suppression: In this experiment, assays were conducted with unselected B6 and B6-Lyb-2.1 spleen cells combined in serial proportions. The reduction in PFC counts caused by mc-α-Lyb-2.1 was directly proportional to the number of B6-Lyb-2.1 cells present, a result that does not fit the idea of a released B cell product inducing suppression.

**Generation of PFC from Lyb-2 Heterozygous Cells in the Presence of Lyb-2 Antibody.** One might argue that a reduction in PFC counts can be caused by attachment of antibody per se during the generation of PFC. The experiment illustrated in Table III speaks against this, for mc-α-Lyb-2.1 caused no reduction in PFC generated from Lyb-2.1/Lyb-2.2 heterozygotes. This result may also imply that PFC reduction depends on blocking of at least most Lyb-2 sites.
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FIG. 4. Evidence against B cell-mediated suppression. B6 and B6-Lyb-2.1 spleen cells were combined in the proportions indicated (abscissa) and cultured with SRBC in the presence of mc-α-Lyb-2.1 (1:50) as before. Data expressed as in Fig. 1 (percent PFC response in the absence of mc-α-Lyb-2.1).

Table III

| Donor of unselected spleen cells | Lyb-2 phenotype | Experiment | Percent response* with mc-α-Lyb-2.1 |
|---------------------------------|-----------------|------------|-------------------------------------|
|                                 |                 |            | 1:50  | 1:100 |
| B6                              | 2/2             | 1          | 116   | 107   |
| DBA/2                           | 1/1             | 1          | 17    | 9     |
|                                 |                 | 2          | 24    | 13    |
| (DBA/2 × B6)F1                  | 1/2             | 1          | 93    | 88    |
|                                 |                 | 2          | 94    | 106   |

* Calculated as for Table I.

PFC Response to T-independent Antigen in the Presence of Lyb-2 Antibody. The following results concern responses to T-independent antigens. Choice of the two antigens TNP-B. abortus and TNP-Ficoll was based on evidence that they represent two categories of T-independent antigen, the former elicits a normal response in mutant B cell-deficient CBA/N mice and the latter does not (11). Table IV signifies that mc-α-Lyb-2.1 did not impede the generation of PFC to either antigen. Because T-independent antigens can be potent stimulators of B cell differentiation, further cultures were set up, as above, and fed 20 μl of mc-α-Lyb-2.1 daily to ensure a continuous sufficiency of Lyb-2 antibody to react with Lyb-2* cells that might be recruited from Lyb-2+ precursors during the culture period; but once again there was no inhibition of the PFC response (data not shown).

PFC Responses of Lyb-2-depleted Spleen Cells. It remains to be asked whether the B cells that generate PFC to T-independent antigen and are not affected by Lyb-2 antibody (Table IV) are in fact Lyb-2+. Table V answers that question. When the responding population is exposed to Lyb-2 antibody and C, rather than Lyb-2 antibody alone (thus eliminating Lyb-2+ cells), the PFC response to both T-independent and T-dependent antigens is equally reduced. We infer that the Lyb-2 molecule is present, but not required, on B cells responding to T-independent antigens.

Clearly, the Lyb-2 molecule is required for efficient generation of AFC in response
**Table IV**

| Antigen* | Experiment 1 | Experiment 2 |
|----------|--------------|--------------|
|          | Concentration mc-α-Lyb-2.1 | Anti-TNP-PFC | Percent response | Anti-TNP-PFC | Percent response |
| TNP-B. abortus (B6-Lyb-2.1) | 1:50 | 4,675 ± 99 | 198 | 3,451 ± 468 | 127 |
|          | 1:100 | 2,445 ± 158 | 104 | 2,347 ± 189 | 87 |
|          | 1:200 | 2,008 ± 142 | 85 | Not done |  |
|          | 1:400 | 2,341 ± 284 | 99 | Not done |  |
| None | 2,361 ± 151 | 100 | 2,727 ± 77 | 100 |
| TNP-Ficoll (DBA/2) | 1:50 | 2,593 ± 108 | 129 | 1,150 ± 91 | 92 |
|          | 1:100 | 2,307 ± 157 | 125 | 1,097 ± 114 | 88 |
|          | 1:200 | 2,307 ± 170 | 125 | 1,250 ± 24 | 100 |
|          | 1:400 | 2,347 ± 209 | 117 | 1,550 ± 94 | 124 |
| None | 2,005 ± 106 | 100 | 1,250 ± 92 | 100 |

10 × 10⁶ unselected spleen cells were cultured with TNP-B. abortus or TNP-Ficoll.

* Spleen donor is in parenthesis.

**Table V**

| Antigen | Mouse strain | Percent response* |
|---------|--------------|-------------------|
| SRBC    | B6-Lyb-2.1   | 31                |
|         | B6           | 92                |
| TNP-B. abortus | B6-Lyb-2.1   | 48                |
|         | B6           | 109               |
| TNP-Ficoll | B6-Lyb-2.1   | 41                |
|         | B6           | 96                |

* Percent response = 100 A/B, where A is the PFC count for cells pretreated twice with Lyb-2.1 antibody plus C, and B is the control count: NMS plus C substituted for antibody plus C.

to T-dependent antigen. Whether it is involved in the process of antigen recognition or in subsequent events, notably, perhaps, in differentiation induced by receipt of a signal from Ly1 cells, remains to be seen. With regard to possible action of α-Lyb-2 on accessory cells, this is unlikely for macrophages generally, because the macrophage population as a whole does not express Lyb-2; nevertheless the possibility that anti-Lyb-2 might react with a minor accessory Lyb-2* population that has escaped serological recognition is presently being studied by functional assay. In any event, Lyb-2 need not necessarily be envisaged as itself the receptor, for it might be part of a receptor molecular complex small enough to be obstructed by Lyb-2 antibody.

**Summary**

The Lyb-2 cell-surface alloantigens of the mouse are selectively and perhaps exclusively expressed in the B lymphocyte lineage, but not on antibody-forming cells. Thus if the Lyb-2 molecule is concerned in specific B cell function, it must participate in the generative phase of the antibody response.

Accordingly, monoclonal Lyb-2 antibody was found to depress the plaque-forming cell (PFC) response to sheep erythrocytes in 5-d Mishell-Dutton assays when added
within the first 3 d of culture, but not later. The rate of PFC generation was not affected, signifying an absolute reduction in the number of PFC generated. Because reduction of PFC counts by Lyb-2 antibody was not affected by exclusion of Lyt-2+ T cells, it is unlikely that the reduction depends on augmented suppression by T cells. Augmented B cell-mediated suppression is also unlikely, because the PFC response of serial combinations of congenic Lyb-2.1 and Lyb-2.2 cells, in the presence of monoclonal Lyb-2.1 antibody, was reduced only in direct proportion to the number of Lyb-2.1 cells present. The PFC response of Lyb-2.1/Lyb-2.2 heterozygous cells was not reduced by Lyb-2.1 antibody, presumably because generation of PFC is impeded only if most Lyb-2 sites are blocked. Further evidence that the molecule identified by Lyb-2 plays a critical role in the generation of antibody-forming cells (AFC) in response to T-dependent antigen comes from the finding that Lyb-2 antibody does not reduce the PFC response to the T-independent antigens trinitrophenylated (TNP) Brucella abortus and TNP-FicolI, although elimination of Lyb-2+ cells from the starting population by Lyb-2 antibody and complement reduces the PFC response to T-dependent and T-independent antigens alike.

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References

1. Sato, H., and E. A. Boyse. 1976. A new alloantigen expressed selectively on B cells: the Lyb-2 system. Immuno genetics. 3:565.
2. Sato, H., K. Itakura, and E. A. Boyse. 1977. Location of Lyb-2 on mouse chromosome 4. Immunogenetics. 4:591.
3. Taylor, B. A., and F.-W. Shen. 1977. Location of Lyb-2 on mouse chromosome 4: Evidence from recombinant inbred strains. Immunogenetics. 4:597.
4. Shen, F.-W., M. Spanondis, and E. A. Boyse. 1977. Multiple alleles of the Lyb-2 locus. Immunogenetics. 5:481.
5. Tung, J.-S., J. Michaelson, H. Sato, E. S. Vitetta, and E. A. Boyse. 1977. Properties of the Lyb-2 molecule. Immunogenetics. 5:485.
6. Yakura, H., F.-W. Shen, E. A. Boyse, and L. Tang. 1980. The Lyb-2 phenotype of hemolytic PFC. Immunogenetics. 10:603.
7. Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (Lond.). 256:495.
8. Mishell, R. K., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med. 126:423.
9. Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology. 14:599.
10. Rittenberg, M., and K. Pratt. 1969. Anti-trinitrophenyl (TNP) plaque assay. Primary response of Balb/c mice to soluble and particulate immunogen. Proc. Soc. Exp. Biol. Med. 32: 575.
11. Mosier, D. E., I. M. Zitron, J. J. Mond, A. Ahmed, I. Scher, and W. E. Paul. 1977. Surface immunoglobulin D as a functional receptor for a subclass of B lymphocyte. Immunol. Rev. 37:89.