

## B-CELL PRECURSORS SPECIFIC TO SHEEP ERYTHROCYTES

### Estimation of Frequency in a Specific Helper Assay

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The complexity of mechanisms underlying an immune response defies effective *in vivo* analysis. *In vitro* analysis, on the other hand, demands not only a system of known and individually variable components, but also the assurance that the course of induction and regulation remains undistorted.

The system proposed by Mishell and Dutton (1) and widely used since, does not quite meet these requirements. Dispersed spleen cells will respond only in media made up with selected batches of so-called supportive fetal calf sera (1). Support, in this context, amounts to an indiscriminate and antigen-independent stimulation of both helper and suppressor T cells (3, 4), rendering the outcome unpredictable. Indeed, under such stimulatory conditions, the number of plaque-forming cells (PFC)<sup>1</sup> arising in Mishell-Dutton cultures may vary by several orders of magnitude, even though controllable variables have been kept constant (2).

The uncontrollable variables are most likely to reside in the T-cell compartment: both the dependence on supportive fetal bovine serum (FBS) and the need for high cell densities ( $\geq 10^7$ /ml) argues for this (2, 4). But the numbers and relative proportions of potential helper and suppressor cells in a spleen are not known beforehand, nor can their degree of arousal by components of the medium be estimated in advance.

In designing a system free from such vagaries, i.e., assuring nonstimulatory conditions, I worked therefore at lower cell densities, eliminated FBS altogether, and supplied an excess of helper cells without raising the level of suppressors. This arrangement allows the study of antigen-dependent induction of B cells or, conversely, may be used for the quantitation of T-cell help.

### Materials and Methods

*Animals.* C57Bl/6 and nude C57Bl/6 (eighth backcross) male or female mice, 6–12 wk of age, were obtained from the Institut für Biologisch-Medizinische Forschung. They were kept under specific pathogen-free conditions behind a sterile barrier, in cages of five.

*Antigens.* Sheep and horse erythrocytes (SRBC, HRBC) originated from individual donors. They were stored in Alsever's solution and washed three times in balanced salt solution (BSS) before use.

*Irradiation.* Irradiation was carried out with a Phillips RT 305 X-ray machine. Mice were given a sublethal dose of 800 rads, cells were irradiated with 3,300 rads.

*Reconstitution of Irradiated Mice and Priming of T Cells.* Within a few hours after whole body irradiation the mice were injected intravenously with  $5 \times 10^7$  syngeneic thymocytes in a 0.4 ml

<sup>1</sup> *Abbreviations used in this paper:* B, bone marrow derived, bursa equivalent; BSS, balanced salt solution; FBS, fetal bovine serum; HRBC, horse erythrocytes; LPS, bacterial lipopolysaccharides; 2-ME, 2-mercaptoethanol; MEM, minimal essential medium; PFC, plaque-forming cell; SRBC, sheep erythrocytes; T, thymus derived; T', primed T-cells; T's, T<sub>H</sub>, T-cells primed to SRBC, HRBC.

vol of BSS. For the priming of T cells  $2 \times 10^6$  washed heterologous erythrocytes were added per 0.4 ml thymocyte suspension. This intravenous injection was preceded 30 min earlier by an intraperitoneal injection of 40 U-USP Heparin (Liquemin Roche, Hoffmann-LaRoche, Basel, Switzerland) in a 0.2 ml vol.

*Culture Conditions.* Two different culture conditions were used: stimulatory culture medium (i.e., culture medium which nonspecifically activates T cells and supports the induction of a primary in vitro antibody response). This culture system is modified from Mishell and Dutton (1) as recently described in detail (culture system B [2, 3]). The medium contains RPMI-1640, supplemented with 20% FBS (selected for its supportive activity),  $5 \times 10^{-5}$  2-mercaptoethanol (2-ME), 1 mM L-glutamine, penicillin, and streptomycin. Nonstimulatory culture medium (i.e., culture medium which does not stimulate T cells and fails to support the induction of a primary in vitro antibody response). This medium was described by Guilbert and Iscove for hemopoietic cell cultures (5) and modified by Iscove and Melchers (6); it is based on Dulbecco's modification of minimal essential medium (MEM), supplemented with additional amino acids, Na-pyruvate, vitamin B12, biotin, and Na-selenite. The medium contains 25 mM Hepes-buffer (Grand Island Biological Co., Grand Island, N.Y., pH 7.3),  $7.5 \times 10^{-5}$  M  $\alpha$ -thioglycerol or  $5 \times 10^{-5}$  M 2-ME, 2.5 gm/l  $\text{NaHCO}_3$ , 100 IU/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. FBS serum is replaced by purified bovine serum albumin (Behringwerke, Marburg Lahn, W. Germany) (1 mg/ml), pure human transferrin (Behringwerke) (10  $\mu\text{g}/\text{ml}$ ) which is 1/3 Fe-saturated by addition of  $\text{FeCl}_3$ , together with a suspension of soybean lipid (pH 7.5, Nattermann & Co., Cologne, Germany) (0.1 mg/ml) and cholesterol (0.05 mg/ml). The lipids are ultrasonically dispersed in 1% bovine serum albumin in bicarbonate-free medium, as described (6).

Cells were cultured either in a 1 ml vol in tissue culture dishes (Falcon 3001) or in a 0.2 ml vol in flat bottom wells of Microtest II tissue culture plates (Falcon 3040) as indicated below.

## Results

*Stimulatory and Nonstimulatory Culture Conditions.* The efficient induction of a primary antibody response in vitro requires supplementation of the culture medium with supportive batches of FBS. The term supportive, coined by Mishell and Dutton (1), is purely operational in the sense that some sera support primary in vitro immunization of murine spleen cells while with others only a few PFC arise. The basis for this difference is controversial (2).

Spleen cells of mice, immunized in vivo several days previously with homologous antigen, give rise to equally high numbers of PFC, whether the medium is supplemented with supportive or nonsupportive serum (1, 2). We have recently found that the purified serum components albumin, transferrin, and lipids, as defined by Guilbert and Iscove (5), and Iscove and Melchers (6), can fully replace serum in a secondary in vitro antibody response.<sup>2</sup>

This is shown in Table I. Under stimulatory culture conditions both normal and in vivo primed spleen cells give rise to high numbers of PFC (22,740 and 40,800 PFC per  $5 \times 10^6$  cultured spleen cells, respectively). Under nonstimulatory culture conditions a drastic difference between the normal and immune spleen cell population is revealed: while unprimed spleen cells respond poorly, the immune spleen cell population gives rise to even higher numbers of PFC under nonstimulatory conditions. Note also that at a lower spleen cell input, normal spleen cells fail to respond, while immune spleen cells give rise to a significant response even at  $2.5 \times 10^6$  cells per ml. Only few PFC arise if the spleen cells are diluted further.

*In Vitro Helper Assay.* In all the following experiments B cells and T cells were added to the cultures separately. B cells were anti-theta-treated spleen cells or spleen

<sup>2</sup> M. H. Schreier. Manuscript in preparation.

TABLE I  
*In Vitro Immunization of Normal and Immune Spleen Cells under Stimulatory and Nonstimulatory Culture Conditions (Anti-SRBC Response Expressed as Direct PFC/Culture)*

Spleen cells/culture	Normal spleen cells		Immune spleen cells*	
	Stimulatory	Nonstimulatory	Stimulatory	Nonstimulatory
$10^7$	47,400	4,360	ND	ND
$5 \times 10^6$	22,740	120	40,800	162,400
$2.5 \times 10^6$	750	0	26,200	73,400

\* Spleen cells were obtained from mice injected 7 days earlier with  $5 \times 10^6$  SRBC i.p.

TABLE II  
*The Requirement of Primed T Cells for the Induction of a B-Cell Response\* (Anti-SRBC Response Expressed as Direct PFC/Culture)*

B cells added per culture	None	$2 \times 10^4$	$5 \times 10^4$
No addition		0	0
$10^6$ T	0	0	0
$5 \times 10^4$ T'	0	$525 \pm 233$	$1,270 \pm 495$
$1 \times 10^5$ T'	0	$510 \pm 70$	$4,840 \pm 1,301$

\* All cultures contained  $5 \times 10^6$  SRBC and the indicated number of T and/or B cells in a 0.2 ml vol of nonstimulatory culture medium. B cells were anti-theta and complement-treated C57BL/6 spleen cells.

cells of syngeneic nude mice. T cells were spleen cells of mice which were irradiated and reconstituted with thymocytes 7 days previously (7). They are termed primed T cells (T') when antigen (SRBC or HRBC) was injected together with the thymocyte suspension or nonprimed T cells (T) when raised in the absence of antigen.

As shown in Table II, under these conditions B cells alone and in combination with T do not give rise to antibody-forming cells. However, in the presence of  $5 \times 10^4$  primed T cells,  $2 \times 10^4$  B cells in a 0.2 ml vol regularly gave rise to PFC. At this low B-cell input the number of PFC cannot be increased by raising the input of helper T cells (Table II), suggesting that the specific B-cell precursors are limiting. At a higher input of B cells more PFC arise and their number can be raised further by providing more helper T cells.

The induction of PFC is fully dependent on antigen (Table III). While we have never observed PFC if antigen was omitted from the culture, T' and antigen occasionally give rise to a few PFC without addition of B cells. The incidence increases with increasing input of T' (up to 8% of the cultures at an input of  $10^5$  T'). The most likely cause is B-cell contamination of the thymocyte preparation used for the reconstitution of irradiated mice. This background can be eliminated by irradiation of the T'-cells, which are known to be radiation resistant (8). X-irradiation decreases the helper activity somewhat, as apparent at lower cell inputs (Table IV). At higher cell inputs we have often observed an increase in helper activity after irradiation. If this were due to the abolition of (radiation-sensitive) suppression, as suggested by others (9), it may be concluded that suppression does not seriously affect our culture system. Irradiation of B cells, however, abolishes the in vitro antibody response. It

TABLE III  
*The Antigen Dependence of the Response*

Addition per culture			PFC/culture*
$2 \times 10^5$ T'	$10^5$ B cells	$5 \times 10^5$ SRBC	
-	+	-	0
+	-	-	0
+	-	+	$28 \pm 28\ddagger$
+	+	-	0
+	+	+	$8,517 \pm 1,236$

\* Each figure is the mean of six cultures.

‡ Two out of the six cultures responded.

TABLE IV  
*Effect of X-Irradiation (3,300 rads) on T'- and B Cells (Anti-SRBC Response Expressed as PFC/Culture)*

	None	$10^6$ Nude spleen cells	$10^6$ X-irradiated nude spleen cells
Nonirradiated T'-cells			
$1 \times 10^6$	0	$6,620 \pm 255$	ND
$2 \times 10^6$	0	$7,119 \pm 810$	0
Irradiated T'-cells			
$1 \times 10^6$	0	$1,800 \pm 679$	ND
$2 \times 10^6$	0	$15,920 \pm 2,036$	ND

TABLE V  
*Lack of Cross-Reactivity of SRBC and HRBC at the T-Cell Level\**

T cells	Antigen	PFC/culture directed against	
		HRBC	SRBC
$10^5$ T	HRBC	0	0
	SRBC	0	0
$5 \times 10^4$ T' <sub>H</sub>	HRBC	$570 \pm 155$	0
	SRBC	0	0
$5 \times 10^4$ T' <sub>S</sub>	HRBC	0	0
	SRBC	0	$670 \pm 127$

\* All cultures contained  $5 \times 10^4$  B cells and  $5 \times 10^5$  of the indicated heterologous RBC.

follows therefore that upon irradiation of the T'-cells all PFC arise from B cells deliberately added to the system.

*Specificity of Helper T Cells.* The specificity of primed helper T cells was assessed by using SRBC and HRBC. These two antigens do not cross-react at the B-cell level and there is evidence against cross-reactivity at the T-cell level as well (10). As shown in Table V, HRBC-primed T cells (T'<sub>H</sub>) do not support an antibody response against SRBC, whereas SRBC-primed T cells (T'<sub>S</sub>) do not help the induction of PFC directed against HRBC. This proves the specificity of the helper T cells. At higher cell density

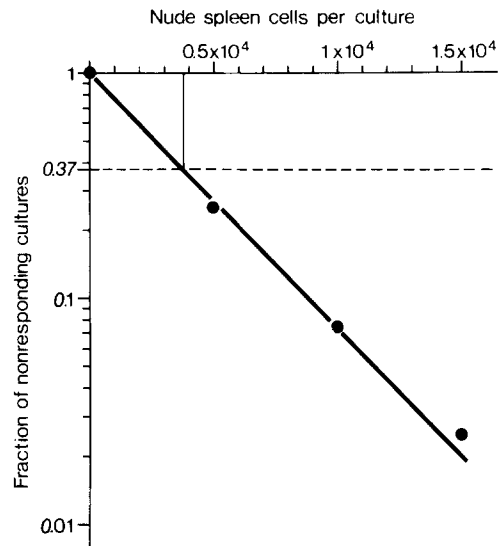


FIG. 1. Limiting dilution analysis of nude spleen cells in the specific helper assay. All cultures contained  $10^6$  irradiated (3,300 rads) T'-cells and  $5 \times 10^6$  SRBC in a 0.2 ml vol of nonstimulatory medium. 5,000, 10,000, 15,000 or no nude spleen cells at all were added per culture (40 cultures per experimental point). PFC were scored 5 days after initiation of the cultures. In the absence of added B cells three wells contained one PFC each. The median number of PFC in the responding wells was 84, 126, and 186 at an input of 5,000, 10,000, and 15,000 nude spleen cells, respectively.

(> $10^6$  cells/ml) and under stimulatory culture conditions some cross-reactivity of SRBC and HRBC can be observed and under these conditions cultures containing T also respond, though at a lower level (data not shown).

*Determination of B-Cell Precursor Frequencies.* The results presented in Tables II-V suggest that this culture system should be suited to limiting dilution analysis of B cells, provided that all cultures contained sufficient helper T cells and antigen. In the experiment shown in Fig. 1, the cultures contained  $10^6$  irradiated T'-cells and  $5 \times 10^6$  SRBC. Without added B cells only an occasional culture gives rise to one or two PFC. In scoring, therefore, cultures containing more than 10 PFC were taken as positive. A saturating input of B cells will give rise to PFC in all cultures. For the B-cell titration shown in Fig. 1, 5,000, 10,000, and 15,000 nude spleen cells were added per culture. The number of PFC was enumerated 5 days later in each individual culture well and the fraction of nonresponding cultures was determined. The frequency of B-cell precursors has been calculated from the zero term of the Poisson distribution. Since 37% of the cultures remain negative at an input of 3,800 nude spleen cells per culture, and since 75% of nude spleen cells are B cells (11), the frequency of SRBC-specific B-cell precursors is about 1 in 2,800.

### Discussion

By excluding uncontrollable variables from the system, a sensitive, specific, and reproducible in vitro helper assay has been developed. The B-cell precursor frequency demonstrated in this system is 20-100 times higher than could be revealed in Mishell-Dutton type cultures (12). These higher frequencies correspond to those found in mitogen-activated B cells (13). The induction of PFC in this system is dependent absolutely on antigen and specific helper T cells.

Two obvious differences between the culture system described above and Mishell-Dutton type cultures are the cell density and the culture medium. These two parameters are to some extent interdependent. Two thresholds of cell density have to be distinguished. The high cell density of about  $10^7$  spleen cells/ml is essential for a primary *in vitro* antibody response. This density requirement may be varied at most two to fourfold, up or down, dependent on the supportive activity of the FBS in the medium and on the source of spleen cells (2). Since this requirement for high cell density can be overcome by supplementing normal spleen cells with *in vivo* primed T cells, we have concluded that its effect is to provide sufficient T-cell help (2). For secondary *in vitro* immunization the required cell density depends on the immunization schedule, but is in most cases still  $>10^6$  spleen cells per ml.<sup>2</sup> A second threshold of cell density becomes manifest at about  $1-3 \times 10^5$  lymphoid cells/ml, even if excessive T-cell help is provided (M. H. Schreier, unpublished observations). This density-requirement has been observed in many culture systems and has been investigated for mitogen stimulation of B-cell cultures; there thymocytes could be used as filler cells to promote growth of single B cells (14).

It is important to realize that the T'-cells fulfill a double function. First, they provide sufficient or saturating T-cell help and, second, they act as filler cells providing a total density of not less than  $2-5 \times 10^5$  cells per ml, as required for growth and maturation of B cells (14). On this reasoning at least  $2.5 \times 10^4$  T'-cells per 0.2 ml culture are needed to allow a response at limiting B-cell input. The frequency of specific helper T' cells is close to  $1 \times 10^{-3}$ , as suggested by preliminary experiments.

The culture medium used throughout these studies has been described recently by Guilbert and Iscove (5). In this medium, serum is fully replaced by the three most abundant serum components: albumin, transferrin, and lipids. Equally important are the other additions to the medium. Some deficiencies of MEM or RPMI medium, which only come to bear at lower cell densities, can be overcome by these supplements to Dulbecco's MEM. This has been observed also in mitogen-induced B-cell cultures (6). With this medium the reproducibility of the described helper assay has been enhanced and consistently higher clone sizes were obtained under conditions of limiting dilution.

The medium of Guilbert and Iscove has been shown to be fully sufficient in promoting proliferation of erythropoietin-sensitive precursors and proliferation and maturation of LPS-reactive B cells. The induction of B cells in the helper assay requires the combined action of two inducers, viz., specific T-cell help and antigen. As judged by microscopic inspection of the cultures, neither antigen nor T cells alone, nor a nonhomologous combination of helper T cells and antigen, was capable of inducing proliferation of B cells. Clusters of proliferating cells arise only if both antigen and T cells of the same specificity are added to syngeneic B cells. The number of proliferating clusters increases with increasing B-cell input.

The results presented in Fig. 1 and Tables II-V show that the plaque output is not proportional to the B-cell input. At high B-cell input (i.e.,  $5 \times 10^4-1 \times 10^5$ ) the number of PFC per input B cell may be an order of magnitude greater than at low B-cell input, i.e., under conditions of limiting dilution. The number of PFC (P) arising after 120 h from the number of total B cells (B) in a culture can be expressed as  $P = f \times \phi B \times 2^{120/d}$  where  $f$  is the fraction of cells in a clone that form a plaque,  $\phi$  B the number of specific B cells ( $\phi \approx 1/3,000$ ) and  $d$  the doubling time in hours. Why is P much smaller than expected at low B input or, inversely, much higher than

expected at high B-cell input? Three possibilities can be distinguished. First, the precursor frequency  $\phi$  might vary, i.e., that the real frequency is higher than 1 in 3,000 but for some reason at lower input cannot be revealed. Though this seems unlikely, the determined precursor frequency  $\phi$  has to be regarded as a minimal estimate. Second, at low B-cell input  $f$  might be smaller, i.e., fewer proliferating cells of a clone mature to secrete antibody and score as PFC. Third, at low B-cell input  $d$  may be larger than at high B-cell input due to some growth promoting contribution from the B-cell compartment. In bacterial lipopolysaccharide (LPS) induced B-cell cultures Andersson et al. (14) found clone sizes of about 80 after 120 h of culture, which amounts to a doubling time of 18 h. This is in line with the clone size observed under limiting dilution conditions in the presented helper assay. To account for a clone size of 1,000 the doubling time would have to be close to 10 h.

The B-cell precursor frequency for SRBC, as determined in this helper assay and in LPS induced B-cell cultures, is very similar. In repeated experiments we found that 1 in 2,500–3,500 B cells of nude and about 1 in 4,000 normal C57Bl/6 mice were specific for SRBC. For LPS and lipoprotein-induced splenic B cells the approximate frequency is 1 in 3,000 and 1 in 5,000, respectively (13). The precursor frequency within the population of mitogen-reactive B cells is close to 1 in 1,000 (13). The precursor frequency demonstrated in this helper assay excludes the possibility, considered by Andersson et al. (13), that the fraction of mitogen-reactive and T-cell help-reactive B cells differed by a factor of 50–100. The question is why the present culture system yields 20–100 times higher precursor frequencies than do Mishell-Dutton type cultures (12)? The most likely reason is that under nonstimulatory conditions sufficient specific T-cell help is provided and this help is not vitiated by the overwhelming suppression induced in Mishell-Dutton cultures (4). Under conditions of the helper assay developed here, the B cells may escape some as yet undefined regulatory mechanisms operating *in vivo* and perhaps also in Mishell-Dutton cultures. The kinetics of the antibody response point in this direction. While PFC arise in the helper assay as expected around day 4 of culture, the number of PFC does not sharply decrease beyond day 5: the majority of cultures keeps scoring high or increasing numbers of PFC up to day 8. The same phenomenon may be observed also in another secondary *in vitro* system which, in contrast to this helper assay, gives rise to indirect anti-SRBC PFC.<sup>2</sup> The common feature of these two systems is low cell density, saturating specific T-cell help, and limiting B cells. Such systems may prove useful in the study of mechanism(s) responsible for the consistent and rapid disappearance of PFC at the peak of the response.

The culture system developed here has the advantage of being unaffected by any mechanism interfering with the induction of helper T cells. In appropriate modifications it can be used to quantitate T-cell help. The system is fully H2 restricted: no PFC arose from H2-incompatible B cells in the absence of syngeneic B cells and allogeneic effects (M. H. Schreier and H. von Boehmer, unpublished observations). Moreover, the system appears applicable to the study of mediators (lymphokines), induced upon specific interaction of helper T cells and antigen.

### Summary

A sensitive, specific, and reproducible *in vitro* helper assay is described which is suited to limiting dilution analysis of murine B cells. 1 in about 3,000 syngeneic

splenic B cells can be induced to form plaque-forming cells (PFC) to sheep erythrocytes in this system. The induction of PFC is absolutely dependent on antigen and specific helper T cells.

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### References

1. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
2. Schreier, M. H., and A. A. Nordin. 1977. An evaluation of the immune response *in vitro*. In B and T Cells in Immune Recognition. F. Loor, and G. E. Roelants, editors. J. Wiley and Sons, Chichester. 127-152.
3. Schreier, M. H. *In vitro* Immunization of Dissociated Murine Spleen Cells. In Research Methods in Immunology. I. Lefkovits, and B. Pernis, editors. Academic Press, Inc., New York. In press.
4. Schreier, M. H., and I. Lefkovits. 1978. Induction of suppression and help during *in vitro* immunization of mouse spleen cells. *Immunology*. In press.
5. Guilbert, L. J., and N. N. Iscove. 1976. Partial replacement of serum by selenite, transferrin, albumin and lecithin in haemopoietic cell cultures. *Nature (Lond.)*. **263**:594.
6. Iscove, N. N., and F. Melchers. 1978. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. *J. Exp. Med.* **147**:923.
7. Miller, J. F. A. P., and G. F. Mitchell. 1967. The thymus and the precursors of antigen reactive cells. *Nature (Lond.)*. **216**:659.
8. Katz, D. H., W. E. Paul, E. A. Goidl, and B. Benacerraf. 1970. Radioresistance of cooperative function of carrier-specific lymphocytes in antihapten antibody responses. *Science (Wash. D.C.)*. **170**:462.
9. Chan, E. L., and C. Henry. 1976. Coexistence of helper and suppressor activities in carrier-primed spleen cells. *J. Immunol.* **117**:1132.
10. Hartmann, K. U. 1970. Induction of a hemolysin response *in vitro*. Interaction of cells of bone marrow origin and thymic origin. *J. Exp. Med.* **132**:1267.
11. Osmond, D. G., and G. J. V. Nossal. 1974. Differentiation of lymphocytes in mouse bone marrow. I. Quantitative radioautographic studies of antiglobulin binding by lymphocytes in bone marrow and lymphoid tissues. *Cell. Immunol.* **13**:117.
12. Quintans, J., and I. Lefkovits. 1973. Precursor cells specific to sheep red blood cells in nude mice. Estimation of frequency in the microculture system. *Eur. J. Immunol.* **3**:392.
13. Andersson, J., A. Coutinho, and F. Melchers. 1977. Frequencies of mitogen-reactive B-cells in the mouse. II. Frequencies of B-cells producing antibodies which lyse sheep or horse erythrocytes, and trinitrophenylated or nitroiodophenylated sheep erythrocytes. *J. Exp. Med.* **145**:1520.
14. Andersson, J., A. Coutinho, W. Lernhardt, and F. Melchers. 1977. Clonal growth and maturation to immunoglobulin secretion "in vitro" of every growth-inducible B-lymphocyte. *Cell*. **10**:27.