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Ontogeny of murine B lymphocytes: Sequence of B-cell differentiation from surface-immunoglobulin-negative precursors to plasma cells*

(induction of surface markers)

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ABSTRACT **Among bone-marrow-derived (B) lymphocytes exist subpopulations of cells that can be induced to express the markers: surface immunoglobulin (Ig), the antigen associated with the immune response gene (Ia), and the receptor for the third complement component (CR). Inducible cells for the first two markers are found in bone marrow, and inducible cells for all three are in spleen. Experiments were designed to determine whether induction involves a single precursor cell population that on triggering with lipopolysaccharide expresses all three surface markers, or three separate precursor cell populations each of which expresses a single marker. Specific B cell subpopulations were eliminated by treatment with anti-Ig or anti-Ia and complement, or by rosette formation with erythrocytes–antibody–complement followed by differential centrifugation, and surviving cells were subsequently tested for inducibility of the three B cell markers. After anti-Ig cytolysis only Ig, but not Ia and CR, could be induced, implying that the Ia- and the CR-inducible cells are Ig^+. Similarly, after anti-Ia cytolysis Ig and Ia but not CR could be induced. Thus, CR-inducible cells must have the Ig^+Ia^+ phenotype. Elimination of CR^+ cells did not affect the induction of Ig, Ia, or CR from their precursors. None of the three elimination experiments affected the conversion of prothymocytes ($Thy\text{-}1^-$) to thymocytes ($Thy\text{-}1^+$). From these results we propose the hypothesis that the differentiation of B lymphocytes proceeds through at least four distinct stages characterized by the following phenotypes: $Ig^-Ia^-CR^- \rightarrow Ig^+Ia^-CR^- \rightarrow Ig^+Ia^+CR^- \rightarrow Ig^+Ia^+CR^+$.**

For many years the study of cell differentiation has been primarily restricted to examination of terminally differentiating cells, i.e., cells that can be easily recognized by their specialized cell products. It has been postulated, however, that each cell lineage is comprised of several precursor cell populations related to each other in precise temporal sequence, and further that each population consists of differentiated cells synthesizing their own "luxury" molecules by which they can be described and assigned to distinct compartments in the cell lineage (1, 2). In reality, distinguishing morphological criteria, such as those accompanying, for example, erythroid maturation or gametogenesis, are the exception for most precursor populations. Our inability to distinguish and isolate precursor populations has made it virtually impossible to analyze fully the developmental history of any cell lineage.

With the discovery that different cell types exhibit unique cell surface antigens (3) it became possible to look for molecular markers on precursor populations, in particular those belonging to the lymphoid series in mice. The hypothesis that lymphocytes express certain surface antigens at defined switchpoints in their differentiation history has been greatly strengthened by the observation that some surface antigens, notably TL, Thy-1, and other thymocyte antigens, can be induced by thymic hormones as well as by other, nonspecific agents (4, 5). Induction experiments of this kind have subsequently been extended to the bone-marrow-derived (B) cell lineage by using the Ia antigen (6) and the complement C3 receptor (CR) (7), and have demonstrated directly the existence of progenitor–successor relationships in committed lymphocyte subpopulations.

In this paper we present evidence for four distinct subpopulations of B precursor cells, based on the induction of unique arrays of the markers Ig, Ia, and CR on their cell surfaces. Furthermore, phenotypic analysis of the four inducible B precursor populations suggests the existence of a sequential relationship between the four B cell compartments. On the basis of these observations a hypothesis is proposed that describes the logical sequence of steps involved in the progressive differentiation of the B cell lineage *in vivo*.

MATERIALS AND METHODS

Mice. Three- to four-week-old $(B6 \times A)F_1$ mice from the Sloan-Kettering colony were used.

Mass Cytolysis. One milliliter of 125×10^6/ml of bone marrow or spleen cells of $(B6 \times A)F_1$ mice was added in the cold to 1 ml of antibody diluted in medium RPMI 1640. Anti-Ig antibody contained 100 μg/ml of purified anti-μ,λ chains and 100 μg/ml of anti-κ chain; control antibody was 100 μg/ml of anti-γ chain [for detail see ref. 6]; anti-Ia^k serum was 1:10-diluted, thymus-absorbed A.TH anti-A.TL serum, and the control serum was A.TL anti-A.TH. One milliliter of 1:10 diluted nontoxic rabbit serum was added as the source of complement (C), and the mixture was incubated at 37° for 45 min. The cells were spun down, resuspended in 35% bovine serum albumin solution (Pathocyte 5, Miles-Pentex, Kankakee, Ill.), and gradient fractionated to yield five layers (6). In the case of Ia treatment of spleen cells a second incubation for 30 min in a fresh addition of C was necessary for complete removal of Ia^+ cells.

Elimination of CR^+ Cells. Cells (20×10^6/ml) of spleen or bone marrow cells of $(B6 \times A)F_1$ mice were mixed with an equal volume of 5% (vol/vol) sheep erythrocytes that were coated with 19S antibody to sheep erythrocytes (Cordis Laboratories, Miami, Fla.) and mouse C up to the C3 component (forming EAC), according to Bianco *et al.* (8). In control experiments erythrocytes were coated only with 19S antibody (EA). The mixtures were incubated at 37° for 30 min in a shaking waterbath, followed by differential centrifugation for 10 min at $15 \times g$ in a refrigerated centrifuge to separate rosettes from free lymphocytes. If residual rosettes were present in the supernatant differential centrifugation was repeated. Free

Abbreviations: LPS, lipopolysaccharide of *Escherichia coli;* C, complement; Ig, surface-associated immunoglobulin; B cell, bone-marrow-derived lymphocyte; T cell, thymus-derived lymphocyte; CR, complement receptor of B cells; SRBC, sheep erythrocytes; EA, erythrocytes coated with antibody; EAC, erythrocytes coated with antibody and C.

* Part II of a series. Part I is ref 6.

Table 1. Effect of pretreatment of (B6 × A)F_1 bone marrow and spleen cells with anti-Ig and C on the induction of Ig^+, Ia^+, CR^+, and Thy-1^+ cells from precursors

C layer cells	Treatment	Percent cells identified by markers and net induction				
		Induction	Ig^+	Ia^+	CR^+	Thy-1^+
Bone marrow	Mixture of anti-IgM (μ) and anti-F(ab)$_2$ (κ)	Without LPS	6 ± 4	16 ± 8	0.8 ± 0.8	17 ± 6
		With LPS	18 ± 4	18 ± 8	0.4 ± 0.8	40 ± 8
		Net induction	12 ± 7	2 ± 4	−0.4 ± 0.8	23 ± 7
	Anti-IgG/Fc (γ)	Without LPS	6 ± 4	18 ± 9	0.2 ± 0.2	11 ± 4
		With LPS	25 ± 5	40 ± 6	1.5 ± 2.1	39 ± 4
		Net induction	19 ± 4	22 ± 6	1.3 ± 2.2	28 ± 6
Spleen	Mixture of anti-IgM (μ) and anti-F(ab)$_2$ (κ)	Without LPS	4 ± 5	18 ± 9	13 ± 7	11 ± 8
		With LPS	20 ± 4	18 ± 10	11 ± 6	44 ± 12
		Net induction	16 ± 10	0 ± 5	−2 ± 3	33 ± 8
	Anti-IgG/Fc (γ)	Without LPS	11 ± 6	17 ± 5	16 ± 8	12 ± 9
		With LPS	28 ± 11	43 ± 5	24 ± 6	39 ± 9
		Net induction	17 ± 13	26 ± 6	8 ± 4	27 ± 4

Bone marrow or spleen populations were treated with rabbit anti-Ig (μ,κ) serum and C, lysing 3–5% of marrow cells and 25–30% of spleen cells. Control antiserum, anti-Fc (γ), and C did not lyse measurable numbers of cells. Treated cells were separated in a discontinuous bovine serum albumin gradient, yielding approximately 10% in the 23%/26% interface ("C layer"). These cells were cultured for 2.5 hr in the presence or absence of 30 μg/ml of LPS, maintaining usually a viability of >85% by trypan blue exclusion. After culturing, subpopulations bearing Ig, Ia, CR, and Thy-1 were enumerated by direct cytotoxicity assay, or rosette assay in the presence of EDTA. Cytotoxicity indices

$$\left(\text{C.I.} = 100 \times \frac{\%\text{ nonviable} - \%\text{ nonviable in C control}}{\%\text{ viable in C control}}\right)$$

are listed, with standard deviation calculated from three to five independent experiments. Net induction is the difference between values from cultures with and without LPS.

lymphocytes (<1% rosettes) were pelleted at 100 × *g* and resuspended in medium to 20 × 10^6 lymphocytes per ml. To lyse erythrocytes, 4 volumes of distilled water were added, followed after 20 sec by 1 volume of 4.25% NaCl. The cells were then spun down, washed once in medium RPMI 1640, and fractionated on the bovine serum albumin gradient.

Bovine Serum Albumin Gradient Fractionation; Induction Assays; Antisera; Cytotoxicity Assays; and Rosette Assay for CR^+ Cells. These were described in detail (6).

RESULTS

Three independent B cell surface markers, Ig (9), Ia (10–12), and CR (8), have been shown to be inducible (13). The following experiments were designed to determine whether: (*i*) the three components are induced simultaneously on a homogeneous population of B precursor cells, as is the case with thymus-derived (T) cell alloantigens in prothymocyte induction (4), or whether (*ii*) different populations respond to *Escherichia coli* lipopolysaccharide (LPS), each expressing a new marker as a result of induction.

Since homogeneous populations of B precursor cells to date have not been sufficiently purified, we have chosen the indirect method of eliminating the subpopulations defined by the three surface markers Ig, Ia, and CR, and testing in the surviving populations which of the markers appear after induction.

Effect of anti-Ig pretreatment

Bone marrow or spleen populations were subjected to mass cytolysis with anti-μ,κ antibody, or with anti-γ antibody (control). The surviving cells, after gradient separation, were then tested in the induction assay, with LPS as the inducing agent. Net inductions of the following markers were scored: Ig, Ia, CR, and Thy-1 (14). Thy-1 identifies the conversion of prothymocytes to thymocytes, which should not be impaired by selective B cell treatments, and therefore serves as a control to safeguard against nonspecific effects. It is evident that the elimination of Ig^+ cells from both populations completely prevents the conversion of Ia^- to Ia^+ cells, and in spleen populations that of CR^- to CR^+ (Table 1). Bone marrow, in contrast to spleen, contains too few inducible CR^- precursors for reliable induction assays of CR^+ cells. Therefore, the inhibitory effect of anti-Ig treatment on CR induction is best seen with spleen populations. The effect is IgM specific, since treatment with anti-IgG/Fc (γ) does not interfere with the induction of Ia and CR from precursors. In contrast to results for Ia and CR, a normal proportion of Ig^+ cells as well as of Thy-1^+ cells can be induced after anti-Ig or anti-γ pretreatment. We conclude that the Ia-inducible cells [as already reported by us (6)] as well as the CR-inducible cells have the Ig^+ phenotype, i.e., are Ig^+Ia^- and Ig^+CR^-, respectively.

We have noted that after cytolysis with anti-Ig, some Ia^+ cells (assuming that all Ia^+ and CR^+ cells express Ig) escape immune cytolysis. Aside from technical imperfections in the cytolytic procedure, such a failure of lysis could be due to modulation, i.e., shedding of Ig receptors (15), in which case cells denuded of Ig could be expected to regenerate Ig during the ensuing culture period. Resynthesis of modulated surface antigen [see TL (16) or Ig (15)], however, is independent of an inductive stimulus. Since our data show that LPS is required to provoke an increase in cells carrying Ig, resynthesis after modulation does not appear to contribute to the observed increase in Ig^+ cells. Alternatively, populations of Ig^-Ia^+ and Ig^-CR^+ cells may exist, but their classification as B lymphocytes or as members of another cell lineage is not clear. Monocytes that lack Ig have been reported to express Ia (17, 18). In the light of these technical difficulties we have interpreted our results only on the basis of whether or not induction did occur, and have placed

Table 2. Effect of pretreatment of (B6 × A)F_1 bone marrow and spleen cells with anti-Ia and C on the induction of Ig^+, Ia^+, CR^+, and Thy-1^+ cells from precursors

C layer cells	Pretreatment	Induction	Percent cells identified by markers and net induction			
			Ig^+	Ia^+	CR^+	Thy-1^+
Bone marrow	Anti-Ia^k	Without LPS	6 ± 5	16 ± 8	0.3 ± 0.3	12 ± 5
		With LPS	18 ± 7	34 ± 9	0.2 ± 0.3	36 ± 5
		Net induction	12 ± 2	18 ± 10	−0.1 ± 0.3	24 ± 4
	Anti-Ia^s	Without LPS	14 ± 9	15 ± 6	0.1 ± 0.1	15 ± 9
		With LPS	25 ± 8	35 ± 10	0.9 ± 0.2	38 ± 5
		Net induction	11 ± 3	20 ± 4	0.8 ± 0.2	23 ± 7
Spleen	Anti-Ia^k	Without LPS	2 ± 3	10 ± 6	14 ± 6	15 ± 7
		With LPS	14 ± 10	34 ± 10	12 ± 6	44 ± 8
		Net induction	12 ± 8	24 ± 13	−2 ± 4	29 ± 10
	Anti-Ia^s	Without LPS	12 ± 3	27 ± 10	16 ± 7	14 ± 7
		With LPS	30 ± 1	52 ± 10	24 ± 5	38 ± 13
		Net induction	18 ± 4	25 ± 10	8 ± 4	24 ± 11

Elimination of Ia^+ cells was by mass cytolysis with A.TH anti-A.TL antiserum, or (control) A.TL anti-A.TH serum. Experimental procedure and evaluation were as described in the legend, Table 1.

less emphasis on the absolute levels of antigen-positive subclasses of cells in induced and uninduced cultures.

Effect of anti-Ia pretreatment

Bone marrow or spleen populations of (B6 × A)F_1 mice were treated with anti-Ia^k, anti-Ia^s (control), or normal mouse sera (control) and C prior to gradient fractionation and induction. As in the preceding experiments, the markers Ig, Ia, CR, and Thy-1 were scored. The results are summarized in Table 2. It can be seen that the inhibitory effect of anti-Ia^k pretreatment is selective for CR^+ induction in spleen cells. Hence the CR-inducible cells are already Ia^+. Induction of the two other B cell markers Ig and Ia is virtually unaffected by anti-Ia^k pretreatment, as is the induction of thymocytes from prothymocytes (Thy-1^- to Thy-1^+). The inhibitory effect is Ia specific, since neither anti-Ia^s nor normal mouse sera pretreatment impairs the CR^- to CR^+ conversion. We conclude from these experiments that CR^+ cells are derived from Ia^+CR^- precursors, that Ig^+Ia^- cells are derived from Ig^-Ia^- precursors.

Effect of elimination of CR^+ cells

Populations of spleen or bone marrow cells were rosetted with EAC's, and rosettes were removed by differential centrifugation before gradient fractionation and induction. Our results show that the induction of all three B cell markers (Ig, Ia, and CR) is unimpeded by prior elimination of CR^+ cells [see Table 3, and our previous report with reference to the induction of CR^+ from CR^- precursors (7)]. EA-treated precursor populations show no significant differences in inducibility from untreated or EAC-treated populations. These experiments demonstrate that all three B cell subpopulations are derived from precursor cells of CR^- phenotype.

Effect of pretreatment with anti-Thy-1 serum and C

To test whether massive cell death in spleen populations affects the induction of B and T precursor cells, T cells were killed with anti-Thy-1 and C, and induction for B cell markers was subsequently carried out. No bystander effect is discernible, and in all cases virtually similar induction levels are observed as compared to untreated precursor populations.

DISCUSSION

We have shown that among B lymphocytes three distinct subpopulations exist that can be induced *in vitro* to express the Ig, Ia, or CR markers. By elimination experiments with cytotoxic anti-Ig and anti-Ia sera as well as removal of CR^+ cell populations by EAC attachment, the phenotypic conversions of three

Table 3. Effect of pretreatment of (B6 × A)F_1 spleen cells with EAC on the induction of Ig^+, Ia^+, CR^+, and Thy-1^+ cells from precursors

C layer cells	Pretreatment	Induction	Percent cells identified by markers and net induction			
			Ig^+	Ia^+	CR^+	Thy-1^+
Spleen	EAC	Without LPS	15 ± 9	24 ± 12	9 ± 6	20 ± 20
		With LPS	37 ± 17	60 ± 14	13 ± 7	46 ± 23
		Net induction	22 ± 14	36 ± 15	3 ± 8	26 ± 9
Spleen	EA	Without LPS	18 ± 10	34 ± 18	22 ± 10	16 ± 2
		With LPS	38 ± 6	62 ± 13	28 ± 5	49 ± 18
		Net induction	20 ± 5	28 ± 9	5 ± 3	33 ± 16

Elimination of CR^+ cells was by rosette formation with EAC, control treatment was with EA. For experimental procedure and evaluation see legend, Table 1. Antisera were specifically purified rabbit-anti IgM (μ,κ); A.TH anti-A.TL; (A/Thy-1.1 × AKR/H-2b)F_1 anti A strain leukemia ASL_1; rabbit anti sheep erythrocytes, 19S fraction.

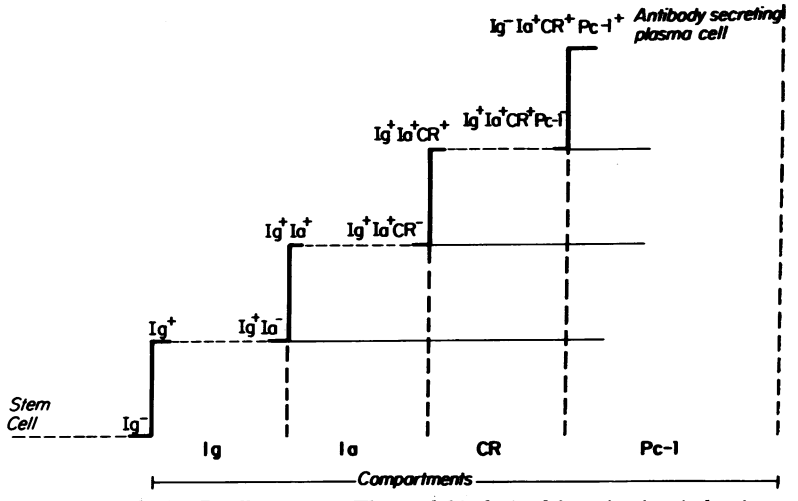

FIG. 1. Model of phenotypic sequences during B cell ontogeny. The model is derived from *in vitro* induction studies, which give evidence for the existence of at least five developmental compartments. It is proposed that the sequential differentiation of B cells through the following compartments—Ig^-, Ig^+, Ia, CR, and Pc-1—is the developmental order in fetal mice as well as the normal mechanism of maintaining the B lineage in adult mice. The differentiation of B cells, as indicated by a concomitant expression of different antigen patterns, is regulated by the microenvironment, which provides inductive stimuli and controls the pool size in the individual compartments. The microenvironment of bone marrow, in contrast to that of spleen, does not appear to support the entire maturation sequence, as indicated by a lack of CR-inducible cells in marrow. Each compartment is made up of both a newly induced and an inducible cell population displaying the same surface phenotype. Inducible cells are genetically programmed to express the next surface antigen in sequence and are poised, waiting for interaction with the external activating agent.

precursor B cell populations to induced populations have been determined as:

(*i*) $Ig^-Ia^-CR^-$ to $Ig^+Ia^-CR^-$ (since this conversion is insensitive to pretreatment with anti-Ig, anti-Ia, or EAC);

(*ii*) $Ig^+Ia^-CR^-$ to $Ig^+Ia^+CR^-$ (since Ia-inducible cells are sensitive to anti-Ig, but not CR (depletion)).

(*iii*) $Ig^+Ia^+CR^-$ to $Ig^+Ia^+CR^+$ (since both anti-Ig and anti-Ia treatments abolish CR induction). There is preliminary evidence to support a fourth conversion relating to the plasma cell antigen Pc-1 (19):

(*iv*) $Ia^+CR^+Pc\text{-}1^-$ to $Ia^+CR^+Pc\text{-}1^+$.

On the basis of these observations we propose a model of B cell ontogeny in which B precursor cells must pass sequentially through a minimum of four compartments in order to become mature CR^+ lymphocytes (Fig. 1). From our experiments at least three compartments are distinguished by the appearance of novel surface markers (Ig, Ia, CR) and a fourth indirectly by the absence of all three markers (Ig^-). Additional compartments and branching side lineages may be defined if new markers are discovered.

In this context induction can be understood as permitting the shift of a precommitted cell from one compartment to the next. The rapidity with which new surface markers appear during induction implies that those cells in bone marrow and spleen that are capable of making the transition preexist in a poised state, each genetically programmed to express (when induced) only the phenotype of the next compartment in sequence. The fact that inducible cells from all postulated compartments in the B lineage exist side by side strongly supports the notion that sequential progression is tightly regulated, since the cells are all presumably exposed to the same environmental induction stimuli and yet are not all expressing the same surface markers simultaneously. Differences in local environments that preferentially support the development of some compartments more than others, as has been described for differentiation of other white blood cells (20), may explain the low frequency of CR inducible cells in bone marrow. It is possible that bone marrow lacks the proper microenvironment for CR reprogramming and that cells attaining the Ia level of differentiation rapidly leave bone marrow to complete their maturation elsewhere. Spleen, on the other hand, may support the entire differentiation sequence from Ig^- to CR^+ cells.

There is now increasing evidence that in preparation for transition from one compartment to the next in a cell lineage DNA synthesis is required (21), the implication being that genetic reprogramming takes place at this time. The appearance of *new* biochemical potential in daughter cells distinguishes that particular cell cycle as being unique from other replicative cycles. It has, therefore, been termed a "quantal cell cycle" by Holtzer and his colleagues to distinguish it as an essential element in the mechanism of cell differentiation. The fact that anti-Ig and C prevent Ia induction but not Ig induction, and that anti-Ia and C prevent induction of CR but not that of Ia suggests that the newly induced Ig^+ or Ia^+ cells are not immediately competent to respond to the inducer and to acquire the next phenotype (i.e., Ia and CR, respectively). It remains to be tested whether DNA synthesis and/or RNA synthesis are required during this refractory period before induction of the next set of markers can occur. The rapidity with which new surface markers appear during the induction step suggests that, as in the case of thymocyte induction, DNA synthesis and cell division are not required for this stage in the phenotypic conversion.

Our model of stepwise B cell differentiation is in agreement with other models, and with observations concerning fetal and neonatal development of the B lineage. B lymphocytes, along with other hemopoietic cells, are thought to descend from stem cells originating in the blood islands of the yolk sac that secondarily populate fetal liver (reviewed in ref. 22). On the basis of Ig expression the first recognizable B cells in mouse fetal liver are found around day 17 of gestation (23), and in the human fetus after the ninth week of gestation (24). These early B cell precursors ("PB" cells) are thought to differentiate stepwise to antigen-responsive B lymphocytes (25). Ryser and Vassalli (26) and Kearney and Lawton (27) have shown that Ig^- B cells of bone marrow acquire Ig in culture, after LPS stimulation, confirming our observation of induction of Ig^- to Ig^+ cells in short term cultures. Furthermore, immature B cells have a CR^-

phenotype, as shown in ontogenetic studies by Gelfand *et al.* (28) and Sidman and Unanue (29). Thus Ig^+CR^- cells precede Ig^+CR^+ cells.

According to our hypothesis, plasmacytes would comprise the ultimate compartment in B cell differentiation. We have based this on the preliminary evidence that Pc-1, an alloantigen of plasma cells, can be induced in spleen populations under experimental conditions similar to those described above, and that the induction is sensitive to pretreatment with anti-Ia and C. In addition, evidence implicating plasma cells as descendants of CR^+ cells follows from functional assays of spleen populations depleted of CR^+ cells in which the primary *in vitro* immune response to SRBC is significantly reduced (30). The immune capacity of the residual CR^- population can be restored by stimulation with LPS. Furthermore, anti-Ia + C pretreatment produces a permanent inhibition of the *in vitro* immune response that cannot be overcome by LPS. This implies that a more extensive differentiation is required for recruitment of antigen-responsive B cells in Ia^+-depleted, as compared to CR^+-depleted, populations. Thus it appears that the interaction of B cells with antigen to initiate an immune response (i.e., differentiation to antibody-secreting plasma cells) can occur only after B precursor cells pass through a number of obligatory compartments in the B lineage and reach the CR compartment. It is possible that contact with antigen prior to the CR^+ stage, but after the cells have acquired the immune receptor (are Ig^+) leads to immunologic tolerance by clonal elimination (31). Such a dualistic behavior of B cells, where antigen triggering of the mature compartment initiates an immune response whereas antigen exposure of an immature compartment suppresses the development of a specific immune response, has been described by Nossal and Pike (32).

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