

THE B CELL REPERTOIRE REVEALED BY MAJOR HISTOCOMPATIBILITY COMPLEX-SPECIFIC HELPER T CELLS

I. Frequencies of a Genetically Defined V Region Marker Among Mitogen- and T Helper Cell-Reactive B Lymphocytes in Normal and Immunized Mice

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The quantitative description of the V gene repertoire in various steady states is of paramount importance not only for our understanding of the laws that govern the selection of the available repertoire from the pool of germ line encoded V genes, but also for predicting the consequences of specific and nonspecific immunological manipulations.

The numbers of cell precursors of clones secreting Ig with a given specificity cannot be directly evaluated in mass cultures or in adoptive transfer experiments, as the number of antibody-producing plaque-forming cells (PFC)¹ or antibody titer depends on both the number of reacting B cells and on clone sizes. Antibody repertoires can therefore only be estimated under limiting dilution conditions in which lymphocytes are either specifically or polyclonally activated. As it has been generally assumed that B cells sensitive to lipopolysaccharide (LPS) express the total repertoire of V region sets on Ig molecules, this ligand has been considered the mitogen of choice for frequency determinations (1).

Recently, however, it has become evident that the repertoire of LPS-sensitive B cells is not completely representative of the total set of Ig specificities in one animal's B cell population and, in addition, it has also been proved that V genes are not, as previously thought, randomly distributed among functional B cell subsets (2, 3). The most important limitation placed on the analysis of the repertoire of LPS-reactive precursor cells, however, is that these lymphocytes may mainly represent newly born short-lived B cells not yet selected into the functional immune system (4-7). This selection may in fact require further differentiation, which enables the cells to become longer living and capable of collaborating with T cells.

In order to obtain accurate information on the set of V genes of cells selected

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¹ *Abbreviations used in this paper:* Con A, Concanavalin A; F6(51), monoclonal anti-M460 antibody; HA, hemagglutination assay; Id, idiotype (idiotypic); Ig, immunoglobulins; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; OVA, ovalbumin; PFC, plaque-forming cell; SRBC, sheep erythrocyte; Th, T helper cell; V, variable (region).

in the network of the functional immune system, it is necessary to study the numbers of precursors of clones of a given specificity among those lymphocytes already capable of responding to T cell-delivered triggering signal. These studies are now possible, as T cell lines capable of delivering help to B cells polyclonally *in vitro* are now available (8, 9), and therefore the V gene repertoire of LPS-sensitive cells can be directly compared to that of T helper-sensitive lymphocytes.

We have recently obtained an alloreactive helper T cell line that recognizes major histocompatibility complex (MHC) antigens. When these helper T cells interact with the appropriate "target-responder" spleen B cells (H-2^d), they activate a large number of these cells to exponential growth and polyclonal Ig synthesis. In the study reported here we took advantage of the triggering property of this helper line and compared the frequency of M-460 id⁺ B cells in LPS- and T helper-sensitive B cell populations in both normal mice and in mice that have been manipulated to various steady states.

The results obtained clearly show that the frequencies of M460 id⁺ clonotypes among LPS- and T helper-sensitive B cells vary considerably, suggesting, therefore, that the repertoire expressed on mitogen-reactive subsets does not necessarily reflect what is expressed in the immune response generated by the same animal's B cell populations.

Materials and Methods

C57Bl/6, BALB/c, CB.20, C3H/He, A/J, B10D2, BAB 25, and SLJ mice were obtained from the Pasteur Institute, Paris, France.

Mitogens. LPS from *S. typhimurium* was obtained from Difco Laboratories (Detroit, MI). Concanavalin A was obtained from the Sigma Chemical Co. (St. Louis, MO).

6B7 Helper Cell Line. The alloreactive 6B7 helper cell line was prepared by culturing 1×10^6 nylon/wool-purified and anti-Ly-2.2- and complement-treated C57Bl/6 spleen cells in RPMI 1640 medium supplemented with glutamine, antibiotics, 10% fetal calf serum, and 2×10^{-5} 2-mercaptoethanol in the presence of 4×10^6 BALB/c irradiated spleen cells. Cultures were restimulated every 4 d for 2 months with irradiated BALB/c spleen cells in the absence of any factor-containing supernatant. After this time, restimulation was carried out in the presence of 15% rat Con A-conditioned medium (CM). The line has now been continuously propagated for 8 months.

Preparation of Con A Supernatant. Conditioned medium (CM) was prepared by culturing 5×10^6 /ml spleen cells from adult rats in the presence of 5 μ g/ml of Con A for 40 h. The culture supernatant was harvested and stored at -20°C . Before use the conditioned medium was always supplemented with 20 mg/ml of α -methyl-mannoside.

Culture Conditions and Frequency Determinations. Anti-theta- and complement-treated spleen cell suspensions were cultured in limiting numbers in RPMI 1640 supplemented with glutamine, antibiotics, 10% fetal calf serum, and 2×10^{-5} 2-mercaptoethanol in the presence of irradiated rat thymus filter cells at a concentration of 3×10^6 cells and either 50 μ g/ml of LPS or the indicated numbers of 6B7 helper T cells. At each cell dose, 24 or 48 replicate cultures were set up and assayed at day 6 for Ig-secretion plaque-forming cells (PFC) by using a staphylococcal protein A plaque assay (10) according to the method described by Pike et al. (11). Alternatively, cultures were continued to days 10–12 and the culture supernatants tested for the presence of M-460 idiotype by a hemagglutination (HA) assay using monoclonal antiidiotype-coated sheep erythrocytes (F6(51)-SRBC) and uncoated SRBC as control. The F6(51) protein was coupled to erythrocytes according to the method of Truffa-Bachi and Bordenave (12). 25 μ l of a 0.25% suspension of F6(51)-SRBC or of uncoated SRBC were mixed in V-shaped microtiter plates with 25 μ l of culture supernatant. After a 2-h incubation at room temperature the number of positive (agglutinated) wells were scored. Wells that agglutinated both SRBC and F6(51)-SRBC

were considered negative. It must be noted that with the HA test used in our experiments the number of SRBC-binding clones was always extremely low, usually too low to allow frequency determination. The specificity and the sensitivity of the assay has been previously described (2). Minimal estimates of precursor frequency were obtained by the minimum chi square method from the Poisson-distribution relationship between the responding cell number and the logarithm of the percentage of nonresponding (negative) cultures.

Antibodies and Immunizations. The F6(51) monoclonal antibody recognizing one idiotope of the M460 id, which is currently expressed in BALB/c mice, was prepared as previously described (13). In some experiments mice were immunized with F6(51) copolymerized with keyhole limpet hemocyanin (KLH) as previously described (13). These mice are referred to as F6(51)-treated mice.

Assay for Functional Activity. Cell proliferation was measured by a 4-h pulse of TdR (1 μ Ci/culture; [3 H]thymidine, 5 Ci/mM, CEA France).

Results

Characterization of the 6B7 Helper Line. The aim of our experiments was to obtain a helper T cell line capable of polyclonally activating target responder B cells in the absence of exogenously added mitogens or antigens. This seemed feasible, since cooperative B cell induction mediated by helper cell recognition against antigen expressed on the B cell surface has already been achieved (8, 9). As we intended to study the expression of the M460 idiotype, which is recurrently expressed in mice with the IghC^a allotypic haplotype (13), in B cells capable of responding to T help we attempted to obtain a helper line capable of recognizing MHC determinants on BALB/c spleen cells. Nylon/wool-purified and anti-Ly-2.2- and complement-treated C57Bl/6 spleen cells were therefore continuously cultured in the presence of BALB/c irradiated spleen cells. 6 wk after the initiation of culture, the cells were harvested and tested for responsiveness and specificity for the stimulator antigens. As shown in Table I, C57Bl/6 cells gleaned from the selective cultures exhibited marked proliferative responses against H-

TABLE I
Specificity of 6B7 Line

Re-sponder cells	Stimulator cells:								3 H TdR uptake
	H-2					Igh-C	Igh-V		
	K	A	E	S	D				
6B7	—	—	—	—	—	—	—	—	<i>cpm</i> 240 \pm 39
BALB/c	d	d	d	d	d	a	a		27,720 \pm 1,050
C57Bl/6	b	b	b	b	b	b	b		310 \pm 45
CB20	d	d	d	d	d	b	b		19,410 \pm 318
A/J	k	k	k	d	d	e	e		419 \pm 39
BALB.K	k	k	k	k	k	a	a		514 \pm 63
BAB.25	d	d	d	d	d	b	a		16,946 \pm 326
B10.D2	d	d	d	d	d	b	b		16,002 \pm 519
B.C8	b	b	b	b	b	a	a		1,224 \pm 415

2×10^4 6B7 cells were cultured in 0.2-ml cultures in the presence of 5×10^5 irradiated (2,000 rad) spleen cells of the indicated strains of mice. 3 H TdR uptake was measured at day 3. The results represent the mean \pm SE of triplicate cultures.

2^d stimulator cells only. As BALB.K and A/J cells did not induce the cells to proliferate, we conclude that the C57B1/6 cells selected by our procedure are most likely specific for determinants encoded in the I-A or I-E region of the MHC. It should also be noted that the addition of conditioned medium to the cultures increased responder cell proliferation without affecting specificity (data not shown) and therefore, in order to obtain large number of cells, the line was continuously propagated in the presence of irradiated stimulator cells and 15% CM.

Functional Activity of 6B7 Line. The next step was to test directly the helper activity displayed by this alloreactive line. The experiments consisted in mixing various proportions of 6B7 cells with anti-theta- and complement-treated BALB/c spleen cells. These cultures were monitored for B cell induction and clonal expansion by measuring numbers of high rate secreting cells in the SpA plaque assay. As can be seen in Fig. 1, we observed the appearance of large numbers of IgM PFC in these cultures and the response was clearly dependent on the number of 6B7 cells added in the assay.

It has been repeatedly reported that one of the distinctive characteristics of T helper cell- and mitogen-dependent B cell activation is the isotype of Ig secreted by the activated B cells (9, 14, 15). In fact a complete dependence of T helper cells for the development of an IgG1 response exists together with a complete dependence of thymus-independent mitogens for the generation of IgG3 PFC. In order to learn whether our helper cells could satisfy this general rule, we studied the isotype distribution of Ig secreted by B cells activated with the 6B7 line. The results (Fig. 2) show that the major isotypes expressed by 6B7 activated B cells are indeed μ and IgG1. Thus our 6B7 C57B1/6 anti-BALB/c cells display all the characteristics of functionally competent helper cells.

Before proceeding to frequency determinations, it was important to ensure

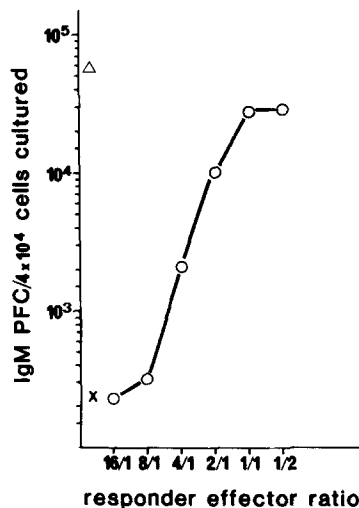


FIGURE 1. 4×10^4 anti-theta- and complement-treated spleen cells of BALB/c mice were cultured in 0.2 ml cultures in the presence of medium alone (x), 50 μ g/ml of LPS (Δ), or the indicated numbers of 6B7 cells (O). The numbers of IgM PFC were determined at day 6 of culture.

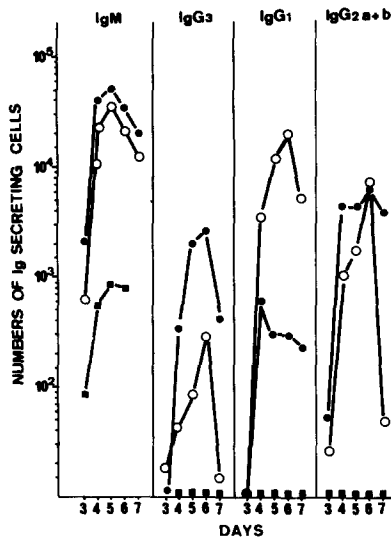


FIGURE 2. 4×10^4 anti-theta- and complement-treated spleen cells of BALB/c mice were cultured in 0.2 ml cultures in the presence of medium alone (■), 50 $\mu\text{g}/\text{ml}$ of LPS (●), or 2×10^4 6B7 cells (○). Isotype-specific PFC were monitored at the indicated days after cultures initiation.

TABLE II
Specificity of the Functional Activity of 6B7 Cells

Stimulator	Responder cells	IgM PFC/culture
—	BALB/c	89
LPS	"	$43,400 \pm 3,100$
*6B7 cells	"	$23,600 \pm 1,800$
—	BALB.K	108 ± 6
LPS	"	$39,400 \pm 5,100$
6B7 cells	"	223 ± 48
—	CB 20	168 ± 69
LPS	"	$38,460 \pm 1,915$
6B7 cells	"	$14,900 \pm 1,810$
—	A/J	104 ± 19
LPS	"	$41,430 \pm 4,140$
6B7 cells	"	160 ± 46
—	C57Bl/6	210 ± 15
LPS	"	$51,490 \pm 4,145$
6B7	"	330 ± 69

* 2×10^4 cells/culture.

2×10^4 anti-theta- and complement-treated spleen cells from the indicated mice were cultured together with LPS or 6B7 cells. IgM PFC were measured after 6 d of culture.

that the specificity of the helper line was also retained at the effector phase. In order to do this, helper cells were cultured with anti-theta- and complement-treated spleen cells from various strains of mice and PFC was measured after 6 d of culturing. Table II shows that only H2^d-positive cells could be induced to

proliferate and secrete high levels of Ig, establishing, therefore, the strict specificity of the functional activity.

Recently, Pobor et al. (16) developed a quantitative assay for detecting effector helper T cells regardless of clonal specificity. As these authors have shown, irrelevant B lymphocytes, i.e., those that do not express either antigen or restriction elements recognized by the effector helper T cells, can also be induced to proliferate and to secrete high rate Ig synthesis in the presence of appropriate concentrations of pokeweed mitogen (PWM) or concanavalin A (Con A). Fig. 3 shows that in the presence of 2 $\mu\text{g}/\text{ml}$ of Con A, 6B7 cells could also effectively cooperate with B cells with an inappropriate H-2 determinant. It is important to note that in the presence of lectin the collaborative response of 6B7 cells to BALB/c cells was selectively inhibited. This point is particularly relevant for our frequency determinations and will be further discussed later.

Frequency of LPS- and Th-Sensitive B Cells. Having established the competence of our helper line to polyclonally activate BALB/c spleen cells we proceeded to evaluate the frequency of every inducible lymphocyte. In the previous section we determined that optimal activation of 4×10^4 cultured B cells was achieved by the presence of 2×10^4 helper T cells in the cultures. In our initial attempt to determine the frequency of 6B7-sensitive B lymphocytes we kept the number of Th cells (2×10^4) constant and limited the numbers of responding lymphocytes. As shown in Fig. 4 this protocol was of little use, as the frequency of IgM-secreting lymphocytes was barely detectable. Varying the numbers of T cells in the assay did not improve the results. The lack of 6B7 cells functional activity in cultures containing limiting numbers of "stimulator responding" lymphocytes probably reflects the inadequacy of low numbers of BALB/c B cells to trigger T

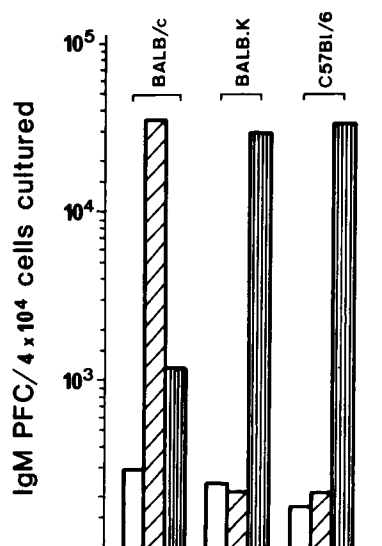


FIGURE 3. 4×10^4 anti-theta- and complement-treated spleen cells from the indicated strains of mice were cultured together with medium alone (□), or 2×10^4 6B7 cells in the absence (▨), or in the presence (▧) of 2 $\mu\text{g}/\text{ml}$ of Con A. The numbers of IgM PFC were measured after 6 d of culturing.

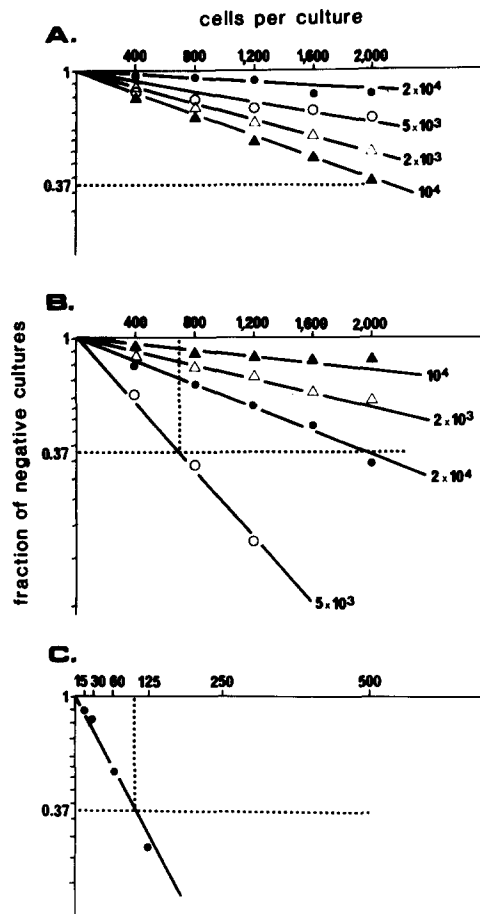


FIGURE 4. The indicated numbers of anti-theta- and complement-treated spleen cells from normal BALB/c mice were cultured in the presence of either 50 $\mu\text{g/ml}$ of LPS (C) or various numbers of 6B7 cells with (B) or without (A) 2 $\mu\text{g/ml}$ of Con A in groups of 24 replicates. IgM-secreting cells were measured 6 d after culture initiation.

helper cells to functional collaboration. This problem could theoretically be resolved by studying the numbers of Th-sensitive precursors in the presence of irradiated splenocytes but, in our experience, the high background of PFC usually observed under these conditions impedes accurate frequency determinations. An alternative approach was to test whether Con A could substitute for the lack of antigen. Fig. 4B shows that the presence of 2 $\mu\text{g/ml}$ of Con A in these limiting experiments considerably improved the number of responding cells. The optimal condition was repeatedly observed with 5×10^3 6B7 cells and lectin. This strict cell dose effect probably reflects a necessary balance between the number of responding cells and the quantity of triggering signals. As the presence of Con A was found to be unnecessary and sometimes even inhibitory when the number of B cells in the cultures increased, we were forced to determine the idiotype frequency and the frequency of every inducible lymphocytes using

different culture conditions. This is clearly a major limitation of the assay, which deserves to be mentioned.

The analyses of the frequencies of idiotype-positive clonotypes have mainly been carried out using the mitogen LPS (2, 17–20). This approach may, however, only have provided information on the idiotypic distribution of a set of immature short-lived cells that have not yet been selected on the periphery of the immune system (6). We therefore attempted a study of the frequency of the M460 clonotype among cells that have already acquired the competence to collaborate with T helper cells.

Fig. 5 shows the frequency of the M460 idiotype in B cells triggered either by LPS or by 6B7 helper cells.

It should immediately be stressed that the system used here does not allow a distinction between Ab3 and Ab1 antibodies (21): i.e., Ig that either recognizes or is recognized by the monoclonal antiidiotypic antibody F6(51). For the sake of simplicity, however, we will refer to the totality of F6(51) reacting molecules as M460-positive Ig, keeping in mind that this is only an operational definition.

The specific frequency determinations among all spleen cells must be related to internal controls for the plating efficiency in the system, namely, for the total frequency of immunoglobulin-secreting clones. This provides the absolute frequencies among the B cell populations studied. From the data in Fig. 5 and that reported in the preceding section we tentatively conclude that in normal BALB/c mice roughly 1/1,000 Ig-secreting LPS-sensitive B cell clones secrete M460 idiotype, while among B cells sensitive to T helper the distribution of this idiotype appears to be much higher, i.e., 1/60–1/100.

The selective preferential expression of M460 id⁺ clonotypes among T helper-sensitive B cells observed in normal nonimmunized BALB/c mice prompted us to study the same frequencies in animals manipulated to various immune steady states. As shown in Fig. 6, the spleen cells of mice immunized 6 months earlier with DNP-OVA show an almost negligible increase in the frequency of both Ig-

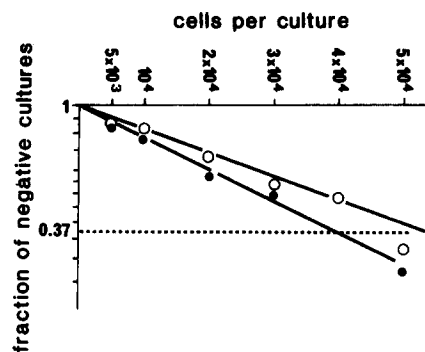


FIGURE 5. The indicated numbers of anti-theta- and complement-treated spleen cells from normal BALB/c mice were cultured in the presence of either 50 μ g/ml of LPS (●) or 2×10^4 6B7 cells (○) in groups of 48 replicates. Cultures supernatants were tested for M460 id-positive Ig 10 d after culture initiation.

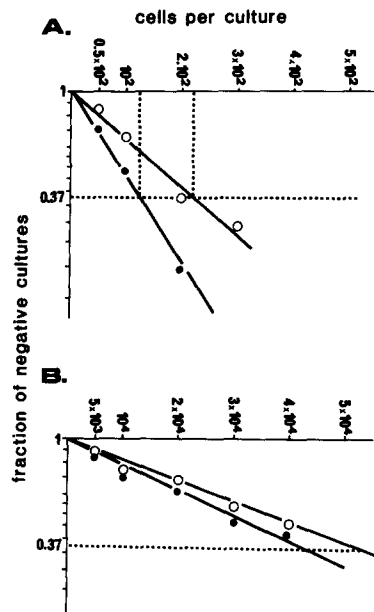


FIGURE 6. The indicated numbers of anti-theta- and complement-treated spleen cells from mice immunized 6 months earlier with 100 μg of DNP-OVA were cultured in the presence of 50 $\mu\text{g}/\text{ml}$ of LPS (\bullet) (A and B) 2×10^4 6B7 cells (\circ) (B) or 5×10^3 6B7 cells and 2 $\mu\text{g}/\text{ml}$ of Con A (\circ) (A) in groups of 48 replicates. The total numbers of Ig-secreting cells (A) or the presence of M460-positive molecules (B) were determined at days 6 and 10, respectively, after culture initiations.

and idiotype-secreting clones (upon LPS stimulation), as compared with that shown by normal mice. The frequency analysis carried out with helper T cells, however, revealed a substantial increase in the numbers of Ig-secreting cells, which was not accompanied by a proportional increase in the number of M460 secreting clones.

BALB/c mice that had been subsequently immunized with antiidiotypic antibodies coupled to KLH and boosted with DNP presented by the same carrier displayed a completely different pattern of precursor frequencies. When limiting dilutions were carried out in the presence of LPS we found only a moderate increase, in both Ig- and idiotype-secreting clones. The precursor analysis of helper-sensitive B cells of the same animals also revealed a moderate augmentation of the internal control, i.e., the number of Ig-secreting clones, but a very strong increase in the frequency of idiotype-positive clonotypes was repeatedly observed. In the helper cell-sensitive compartment of the splenic B cell population of these animals, the absolute frequency of M460 secreting cells was roughly 1/10–1/20. Interestingly we obtained identical results with 129 Sv mice (IgC^a haplotype) even though, as these mice do not have the appropriate H-2 determinant, M460-positive clonotypes sensitive to T helper cells had to be determined in the presence of Con A (data not shown), that as shown in Fig. 3, reveals the functional activity of any helper cells regardless of clonal specificity.

The extremely high frequency of idiotype-positive clones observed in these experiments reconciles the discrepancies that have previously been observed,

and confirmed here, between the high titers of circulating idiotype and the relatively low LPS-sensitive idiotype-positive clonal frequency detected in hyper-immunized mice (17). Our finding, in fact, ascribes the secretion of serum antibodies to T helper responder B cells. We have recently reported that F6(51) copolymerized with LPS induces a substantial M460-positive response (when injected in BALB/c mice), which is completely T independent as it can also be obtained in nude mice (22). We were therefore interested in determining the frequency of T helper-sensitive and LPS-sensitive B cells secreting M460 idiotype in BALB/c mice that have been previously treated with immunogenic doses of F6(51)-LPS. As shown in Table III, the frequency of M460 id-positive B cells did not vary significantly from that in normal mice, whatever the stimulus used to trigger B cells. In addition to this, the frequency of Ig-secreting clones was also only marginally modified, suggesting therefore that the pool of mitogen-reactive cells is indeed composed, as has already been suggested, of short-lived cells that are not selected in the functional immune system.

Discussion

The aim of the present work was to analyze the distribution of a genetically defined V region determinant among splenic B cells sensitive to T helper cell triggering signals. The reason for our interest in this particular B cell subset is that it is likely to be composed of mature B lymphocytes selected in the periphery of the immune system. To this end we used a strategy that uses the functional property of an alloreactive T helper cell line recently selected in our laboratory. This line appears to be specific for I-A^d or I-E^d determinants and when cultured together with "target-responder" B cells of the appropriate H-2 haplotype, induces these B cells to proliferate and differentiate to secrete high levels of Igs in the absence of exogenous ligands.

TABLE III
*Frequencies of LPS- and T Helper-reactive B Cells Producing M460⁺
Positive Ig in Normal Mice and Mice Immunized with F6(51)-LPS*

	Frequency in LPS-sensitive cells	Frequency in Th-sensitive cells
<i>Expt. 1</i>		
Normal BALB/c		
IgM	1/80	1/780
MOPC 460	1/50,000	1/46,000
*F6(51)-LPS BALB/c		
IgM	1/60	1/650
MOPC 460	1/46,000	1/48,000
<i>Expt. 2</i>		
Normal BALB/c		
IgM	1/100	1/600
MOPC 460	1/58,000	1/52,000
F6(51)-LPS BALB/c		
IgM	1/70	1/700
MOPC 460	1/49,000	1/48,000

* Immunized with 10 µg F6(51)-LPS. Mice tested 4 months after immunization.

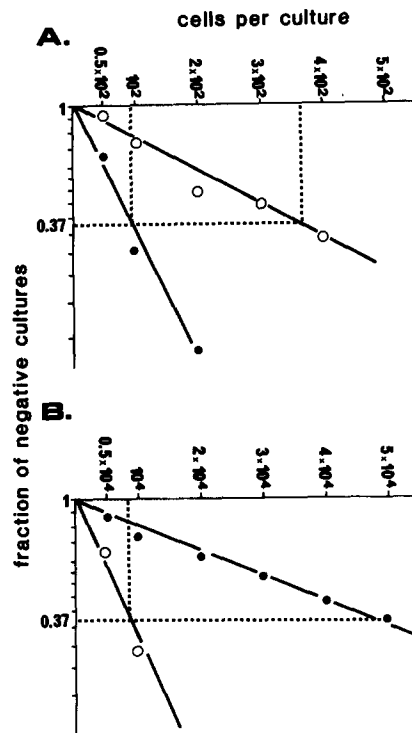


FIGURE 7. Same as Fig. 6 but the mice had been immunized 6 months before the test with $50 \mu\text{g}$ of F6(51) KLH in CFA and boosted with $50 \mu\text{g}$ of DNP-KLH.

Although the helper cells used in these studies were not cloned, they appear, as judged by their selective functional activity, to be very restricted in their specificity. In particular, 6B7 cells failed to activate BALB.K and A/J B cells, while vigorous polyclonal secretion was repeatedly obtained with CB20 and BAB.25 splenic B cells. This seems to exclude antigens other than H-2-encoded determinants as the target of 6B7 recognition.

Using normal culture conditions i.e., 4×10^4 responder cells, optimal responses required an effector/responder ratio of $\frac{1}{2}$, higher numbers of T cells being either inhibitory or having no effect. 2×10^4 6B7 cells also induced optimal responses in cultures containing as few as 5×10^3 B lymphocytes. For our experiment it was also essential to determine the total frequency of Ig-secreting cells and we had to carry out analyses in which the numbers of B lymphocytes seeded in the cultures ranged from 2×10^3 to 10^2 . Under these conditions, however, polyclonal help was barely detectable at any T cell input tested. This problem was easily obviated by adding Con A to the cultures. The possibility that Con A acts by inducing the release of B cell growth factor from rat thymus filler cells seems unlikely, since first, we could never detect an Ig response in cultures containing B cells, Con A, and filler cells in the absence of 6B7 lymphocytes (data not shown), and second, Con A could bypass H-2 restriction even in cultures that did not contain filler cells (Fig. 3). Thus we believe that under these conditions the lectin requirement reflects the necessity of the T cell

triggering signal, which obviously cannot be provided by the low numbers of B cells seeded in these cultures. The role of Con A is therefore either to trigger directly the helper cells or to favor T-B cell contact, which is the necessary requirement for cooperative responses.

The absolute frequencies of a given specificity can only be determined by relating the total frequency of Ig-secreting clones to the specific frequency determination. As we could not quantify these two parameters by the same experimental conditions, the first requiring lectin, the second being lectin independent, we are aware that the absolute frequencies obtained here should be considered as preliminary and subjected to further improvement pending the development of culture conditions that will allow a uniform analysis of the two parameters.

H-2 determinants are expressed on the totality of B cells and therefore our expectation was that our helper line would trigger every single B cell. Surprisingly, this was not the case. On the contrary, the frequency of T helper-sensitive B cells was found, in our experimental conditions, to be substantially lower than that of B cells sensitive to LPS. Similar, although less marked differences, have been reported by Augustin and Coutinho (9), who studied the frequency of inducible B cells sensitive to the helper T cells specific for minor antigens expressed on the B cell surface. The most likely explanation for the relatively low frequency observed is that the spleen is the major site of migration of newly formed B cells (23, 24) and is therefore likely to be populated by large numbers of lymphocytes that have not yet acquired sensitivity to T help. These results contrast with those of others who reported that the "quasi" totality of LPS-sensitive cells could be restimulated *in vitro* by helper T cell products (25, 26). However, these experiments do not exclude the likely possibility that the B cells studied may have further differentiated during the primary cultures.

Studies aimed at understanding the rules by which the receptor repertoire expressed on B cells is selected in the immune system have been previously carried out in several laboratories (2, 17-20, 27-30). The general strategy used was to compare the frequencies of LPS-sensitive cells positive for a given idiotype in different organs or in the splenocytes of adult and newborn animals. The outcome of these various studies are remarkably concordant, namely that idiotype-specific clones are neither positively nor negatively selected from the point of appearance in the bone marrow until they arrive in the spleen. These results were quite surprising as they preclude any mechanism of selection of the available repertoire from the pool of germ line encoded genes in the absence of antigen. As at any point in its lifetime, an individual expresses only a tiny fraction of his potential repertoire, an immune system without selection would necessarily lead to a repertoire based upon probabilistic recombinatorial and mutational events. This would indeed be a very inefficient system, as the majority of clones would never have the chance to be expressed. The data reported here provide a solution to this apparent paradox. The frequency of M460-positive clones in normal mice was found to be 1/1,000-1/2,000 among LPS-sensitive cells, while the distribution of the same clonotype in the B cell compartment responsive to T cell help was at least 10 times higher. The most reasonable conclusion is that the V gene repertoire of LPS-reactive cells is not representative of the selected

available repertoire, but rather reflects the sets of antibody V region generated by random recombination of VDJ genes as they occur during early differentiation in the B cell lineage. This interpretation of our results is consistent with several reports that show that during ontogenesis only certain fractions of the newly generated (LPS-reactive) B cells become responsive to T cell help and enter the pool of long-lived recirculating B cells (4, 31–32).

Recently Rajewsky and Takemori (33) reported convincing evidence that dovetails with our conclusion. Animals that had been neonatally suppressed for the idotype Ac38 mounted a normal anti-NP response when immunized with NP coupled to chicken gamma globulins but did not express the Ac38 marker. Surprisingly, however, limiting dilution analysis of LPS-reactive precursor cells revealed that the frequency of Ac38⁺ precursors in the suppressed mice was identical to that of the control untreated animals. We can provide another example along these lines. The frequency of M460 idotype-positive clones in mice immunized with F6(51)-KLH and boosted with DNP coupled to the same carrier was much more affected (as compared with that observed in normal mice) among the T helper-sensitive B cell subsets than in the pool of LPS responsive lymphocytes. The differences in clonotype frequency between the two B cell populations were in effect quite striking i.e., 1/10–1/20 for the former and 1/600–1/800 for the latter. These results provide also an explanation for the previously observed disproportion between the high titers of circulating antibodies and the low precursor frequencies generally observed in these immunized mice. On the other hand the finding that the M460 idotype-positive clones were only marginally increased in animals immunized with DNP-OVA (as compared with normal mice) seems to correlate well with the low percentage of idotype-positive molecules generally observed in the pool of BALB/c anti-DNP antibodies. One question of major interest concerns the specificity of Th-sensitive 460 Id⁺ precursors, as these clones can be either composed of true Ab1 DNP binding 460 Id⁺ cells or by cells bearing the 460 Id, but with different antigenic specificity. The answer to this question, however, requires the availability of a very sensitive radioimmune assay by which the culture supernatants can be screened for the presence of Igs that simultaneously react with DNP and F6(51) monoclonal antibodies. Unfortunately our repeated attempts to reveal the presence of Igs that reacted with insolubilized DNP-BSA by ¹²⁵I-labeled F6(51) have repeatedly failed. Therefore, at the present time, the question of the nature of Th-sensitive 460 Id⁺ clones cannot be resolved.

The majority of the studies reported here were purposely carried out on animals immunized with thymus-dependent antigens. It was interesting to assess, however, whether the V region repertoire of T helper-sensitive B cells could be influenced by immunization with thymus-independent antigens. The results obtained with F6(51)-LPS immunized mice show that this is clearly not the case, suggesting therefore that the cells involved in this type of immune response are short-lived lymphocytes that undergo fast terminal maturation.

We have made above a strong case for the necessity of a mechanism responsible for the selection of the available repertoire. We have also presented evidence that suggests that the clones selected from the periphery of the functional immune system are those that have acquired the functional property of respond-

ing to T helper cell signals. It is therefore tempting to conclude that the selection of the available repertoire is mediated by T helper cells. If this is the case it would necessarily follow that nude mice lack the basic property of selecting their own V genes from the periphery of their immune system and, as argued above, their repertoire should (at least) differ from that of normal mice. As, on the contrary, all the available evidence converges to indicate that nude mice have a normal and functional repertoire (22, 34), we believe that the mechanism of V gene selection is independent from T cells and that the acquired property of B cells of responding to T helper cell signals is only a differentiation marker that is acquired independently from the presence of helper cells. Although the capacity of B cells from nude mice of responding to T cell-delivered signals is well documented (35) and fully supports this view, the final explication of the role of T cells in the selection of the available repertoire will only come from studies similar to those reported here, using B cells of athymic animals.

In conclusion, our data ascribe a different distribution of sets of genetically defined antibody V region determinants to the LPS- and T helper-sensitive B cell compartments. The first compartment seems to include precursor distribution as it occurs during the early stage of B cell development (potential repertoire) and it most likely depends on the number of genes involved in the expression of each specificity; the second appears to include the V region repertoire expressed at a later stage of B cell ontogenesis (functional repertoire) and depends on various mechanisms of selection. The possibility of studying these two B cell compartments separately both in mice and humans (36) opens, therefore a new approach to the analysis of several still unresolved issues, in particular, the cellular basis of clonal dominance, the contribution of somatic mutants to the available repertoire, and the mechanism of the germ line V gene selection in the functional immune system.

Summary

The aim of the present work was to analyze the frequencies of a genetically defined variable (V) region marker in the B cell subset sensitive to T cell help. To this end we used an alloreactive T cell line that has the property of inducing B cells of the appropriate haplotype to exponential growth and polyclonal antibody synthesis. The frequency obtained with this helper line was also directly compared to that obtained with lipopolysaccharide (LPS). We found that in normal BALB/c mice the frequency of M460-positive clonotypes was respectively, 1/100 and 1/1,000 among the T helper- and LPS-sensitive B cell subsets. In mice immunized with antiidiotype coupled to a thymus-dependent antigen, the differences in the numbers of idiotype-positive precursors were even more accentuated, i.e. 1/20 in the B cell subset triggered by T helper cells and 1/800 in those cells responsive to LPS. The frequencies of the M460 determinant in mice immunized with anti-idiotypes coupled to thymus-independent antigens were not significantly different, in either B cell subset, from those obtained with spleen cells of normal nonimmunized animals. Taken as a whole, our results imply that the V gene repertoire revealed by LPS includes precursor distribution, as this distribution occurs during the early stage of B cell development (potential repertoire), while the repertoire revealed by T helper cells includes the V region

distribution of those clones that are selected in the periphery of the functional immune system.

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