

DELETION OF b5 IMMUNOGLOBULIN-BEARING LYMPHOCYTES IN ALLOTYPE-SUPPRESSED RABBITS

BY MICHAEL R. HARRISON, ROSE G. MAGE, AND JOSEPH M. DAVIE*

(From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014)

(Received for publication 27 September 1972)

In the rabbit, *b* locus allotypic determinants present on kappa-type light chains have been demonstrated both on circulating immunoglobulins (1, 2) and on the membrane-associated immunoglobulins of bone marrow-derived (B)¹ lymphocytes (3-6). The production of circulating b5 immunoglobulin (Ig) can be suppressed by exposing rabbits in utero or at birth to anti-b5 antisera (allotype suppression) (7-10). Only after a period of weeks to years do these allotype-suppressed rabbits spontaneously escape and begin making small amounts of the suppressed b5 Ig. Although the dynamics of suppression and recovery of circulating b5 Ig have been well described (10), lymphocytes with b5 Ig on their membrane have not been directly studied in these rabbits. Sell found a reduced blastogenic response to specific antiallotype antisera in allotype-suppressed rabbits (11), providing indirect evidence that lymphocytes bearing the suppressed allotype on their membranes may be affected.

The mechanism of allotype suppression is unknown. Like the more recently described suppression of Ig allotypes in mice (12, 13), Ig classes in chickens (14) and mice (15), and Ig idiotypes in mice (16) by appropriate anti-Ig antisera, allotype suppression in the rabbit is presumably the consequence of interaction of the suppressing antibodies with Ig determinants present on the surface of small lymphocytes or their precursors. We have employed sensitive techniques of autoradiography and fluorescence microscopy to directly document the deletion of b5-bearing peripheral blood lymphocytes (PBL) in rabbits fully suppressed for b5, providing evidence that suppression operates at the level of Ig-bearing precursor cells.

Spontaneous recovery from allotype suppression offers a unique opportunity to study the dynamic relation between the appearance of lymphocytes with b5 on their membrane and the appearance of circulating b5 Ig, and may provide insight into the process by which Ig-bearing lymphocytes mature into Ig-secreting cells. We have studied the appearance and recovery of b5-bearing lymphocytes in relation to secreted b5 Ig after escape from allotype suppression. These

* Present address: Department of Pathology and Microbiology, Washington University School of Medicine, St. Louis, Mo. 63110.

¹ *Abbreviations used in this paper:* B cells, bone marrow-derived lymphocytes; FCS, fetal calf serum; HAI, hemagglutination inhibition; Ig, immunoglobulin; MEM, minimal essential medium; mg N/ml, milligrams of nitrogen per milliliter; PBL, peripheral blood lymphocytes.

studies provide evidence that in escape from suppression, the production of b5 Ig is dependent on the presence of b5-bearing lymphocytes, but that during recovery from suppression the presence of adequate numbers of b5-bearing lymphocytes does not insure the production of commensurate amounts of circulating b5 Ig.

Materials and Methods

Experimental Animals.—Rabbits used in these experiments were of known parentage and allotype from closed colonies bred at the National Institutes of Health. Sera and antisera were characterized as previously described (17).

Allotype Suppression with Anti-b5 Antisera in b5b5 Homozygous Rabbits.—Treatment of neonatal b5b5 rabbits with anti-b5 antisera is complicated by the presence of b5 Ig derived in utero from the mother. The presence of maternally derived b5 Ig was circumvented in two ways. Originally b5b5 zygotes were transferred to b4b4 foster dams (18). Since they received only b4 Ig in utero they could be suppressed with anti-b5 antiserum at birth (19). Littermates H206-1-2, and-3 were suppressed in this way. Once b5-suppressed homozygous b5b5 females had matured, mating to normal b5b5 bucks produced offspring raised in mothers free of b5 Ig who could thus be suppressed at birth with anti-b5 antisera. Litters H2, K326, and K165 were suppressed in this way. All suppressed rabbits were given 5 ml of b4 anti-b5 antisera (raised in b4 rabbits by immunization with b5 IgG) intraperitoneally at birth and once or twice weekly for up to 13 wk.

Four litters of homozygous rabbits in various stages of suppression and recovery were studied over a 6 mo period. Nonsuppressed littermates (in the case of litter K165) or age-matched b5b5 rabbits acted as controls.

Quantitation of IgG and b-Locus Allotypes in Sera.—Sera were assayed for total concentration of IgG as well as concentrations of b4 and b5 IgG by radial diffusion in gels as previously described (20). Because the lowest concentration of b5 IgG detectable by radial diffusion is $\sim 100 \mu\text{g/ml}$, a hemagglutination inhibition assay which detected $1 \mu\text{g/ml}$ of b5 IgG was also employed. The procedure was similar to that described previously (21) except that sheep red blood cells were sensitized with b5 Ig by the chromic chloride method (22) and 5% fetal calf serum (FCS) was used as diluent in the microtiter assay. Immunoelectrophoresis was performed as previously described (23).

Preparation of ^{125}I - and Fluorescein-Labeled Specific Antiallotype Antibodies.—Antisera were produced by immunization of homozygous b6 and b9 rabbits with purified b4 or b5 IgG prepared from pooled sera as previously described (17). The preparation of specific anti-allotype antibody from similar antisera and the labeling with ^{125}I and fluorescein have been previously described (6). Antibodies were specifically purified by using solid immunoadsorbents prepared by coupling purified IgG of either b4 or b5 allotype to agarose beads (24).

The purified antibodies were labeled with ^{125}I by the chloramine-T method (25). Specific activities were 23–34 $\mu\text{Ci}/\mu\text{g}$ and final concentrations of antibodies were 5–10 $\mu\text{g/ml}$. Fluoresceinated antibodies were prepared (26) in high concentrations (10 mg/ml) in phosphate-buffered saline and required no additional absorption before use.

The antibodies purified as described were specific and non-cross-reacting when tested for precipitation by double diffusion in agar gels and when used to label lymphocyte preparations from b4 and b5 homozygous rabbits.

Isolation of Rabbit Peripheral Blood Lymphocytes and Detection of Membrane-Associated Ig Allotype with ^{125}I and Fluorescent Antiallotype Antibodies.—Membrane-associated Ig allotypes were detected on PBL by autoradiography and fluorescence microscopy as previously described (6). Erythrocytes in heparinized blood samples were sedimented with methyl cellulose-

Hypaque and lymphocytes purified over Ficoll-Hypaque as described (6). Residual erythrocytes were lysed with hypotonic NH_4Cl solution. Cells were washed and resuspended at 4°C in minimal essential medium (MEM) supplemented with 10% FCS and heparin (50 U/ml). Approximately 1×10^6 nucleated cells containing greater than 90% lymphocytes were isolated from each milliliter of peripheral blood.

$1-10 \times 10^6$ lymphocytes were suspended in 0.2 ml of MEM containing heparin and sodium azide (1 mg/ml), and incubated with 25-100 ng (5-10 μl) of ^{125}I antiallotype antibody or 0.1-0.2 mg (10-20 μl) of fluorescent antiallotype antibody at 4°C for 30 min. For ^{125}I labeling, cells were washed four times through FCS and examined by autoradiography after exposure *in vacuo* for 2-6 days. Although many cells were more densely labeled, those cells with at least three silver grains in contact with the cell were scored as positive. Only cells with the morphological characteristics of lymphocytes using methyl green-pyronin stain were considered. Lymphocytes incubated with fluorescein-conjugated antiallotype antibodies were washed once through FCS and examined using a standard optical system for fluorescence (Leitz BG 12 + KP 495 excitation filters, K 530 barrier filter).

Since labeling of viable lymphocytes was carried out at 4°C in the presence of azide, only membrane-associated Ig determinants were detected. Most cells labeled with fluorescein-conjugated antiallotype antibodies under these conditions revealed a patchy distribution of determinants.

RESULTS

Rabbits Completely Suppressed for Circulating b5 Had No Peripheral Blood Lymphocytes with Membrane b5.—Of seven K165 littermates, five received repeated injections of anti-b5 from birth to 13 wk of age and two were kept as untreated normal controls. From 4 to 10 mo of age, the PBL of these rabbits were examined for membrane b5 and the sera assayed for circulating b5 and total IgG. Table I summarizes the data for the period 4-7 mo of age when the rabbits were completely suppressed. In the five treated littermates there was no detectable circulating b5 and likewise no PBL with membrane b5. Total IgG concentrations in these suppressed rabbits were quite normal and not significantly different than littermate controls by virtue of compensatory production of immunoglobulins with lambda-type light chains (19, 27). During this same period the two untreated littermate controls had normal levels of circulating b5 (expressed both as concentration and as percent of total IgG) and normal levels of b5-bearing peripheral blood lymphocytes. The percent of b5-bearing lymphocytes ranged from 13 to 43% of PBL with a mean of 25.4 in these two normal rabbits; these values are typical of the relatively wide range found in normal b5 rabbits (10-44% with mean \pm SEM of 24.2 ± 2.8).

When Rabbits Spontaneously "Escaped" from Suppression, the Appearance of Detectable Circulating b5 Was Heralded by the Appearance of Lymphocytes with Membrane b5.—Rabbits suppressed by anti-b5 treatment usually escape from suppression and begin producing circulating b5 Ig within the 1st yr of life. Regular examinations of a litter suppressed for b5 during the period when spontaneous escape from suppression occurred demonstrated that the appearance of circulating b5 was temporally linked to the appearance of b5-bearing lymphocytes. Fig. 1 presents graphically the status of serum b5 and lymphocyte

TABLE I
Total Deletion of Lymphocyte Membrane b5 and Serum b5 in Rabbits Completely Suppressed for b5 (Age 4-7 mo)

Littermates	Total IgG*	b5 IgG*	b5‡	PBL with membrane b5§
	mg N/ml	mg N/ml	%	%
Suppressed with anti-b5 at birth				
K165-2	0.68 ± 0.01	<0.01	<2.0	<0.1
K165-4	0.78 ± 0.04	<0.01	<2.0	<0.1
K165-5	0.53 ± 0.06	<0.01	<2.0	<0.1
K165-6	0.58 ± 0.03	<0.01	<2.0	<0.1
K165-7	0.60 ± 0.02	<0.01	<2.0	<0.1
Untreated littermates				
K165-1	0.56 ± 0.03	0.53 ± 0.02	96.0 ± 4.8	29.0 ± 7.9
K165-3	0.55 ± 0.02	0.52 ± 0.01	94.8 ± 2.6	15.0 ± 2.0

* Mean ± SEM of 10 serum samples collected every 1-2 wk from age 4 to 7 mo for each rabbit. No b5 IgG was detectable in the suppressed rabbits. The lowest concentration detectable by radial diffusion is ~0.01 mg N/ml, or <2% of the total IgG concentration.

‡ Percent b5 relative to total IgG = $\frac{\text{b5 mg N/ml}}{\text{IgG mg N/ml}} \times 100$.

§ Percent of peripheral blood lymphocytes with membrane

$$\text{b5} = \frac{\text{no. of lymphocytes labeled with } ^{125}\text{I anti-b5}}{\text{total no. of lymphocytes counted}} \times 100.$$

Values are mean ± SEM of two to eight values for each rabbit. In suppressed rabbits no b5-bearing cells were found in counting >1,000 cells on each slide.

membrane b5 in five allotype-suppressed rabbits (K165-2, -4, -5, -6, -7) and their untreated littermate controls (K165-1, -3) from age 4 to 10 mo. During this time the suppressed rabbits escaped and began producing b5. In Figs. 1 *a* and *b*, b5 and total IgG concentrations are plotted to demonstrate that in normal rabbits serum b5 follows closely the normal fluctuations in total IgG concentrations (Fig. 1 *b*), whereas in suppressed rabbits, serum b5 concentrations are low and IgG concentrations are normal (Fig. 1 *a*). In Figs. 1 *c* and *d* serum b5 is expressed as percent of total IgG. Before 7 mo of age, the b5-suppressed rabbits had no detectable circulating b5 (Figs. 1 *a* and *c*) and no b5-bearing PBL (Fig. 1 *e*). Then b5-bearing lymphocytes appeared in small numbers (3-9% of cells counted) for the first time at 7¼ mo (Fig. 1 *e*), at a time when there was still no b5 in serum detectable by radial diffusion (Figs. 1 *a* and *c*). All suppressed rabbits began producing small but detectable amounts of circulating b5 Ig between 7½ and 8½ mo. These low levels of b5 Ig are shown on a magnified scale in the inserts of Figs. 1 *a* and *c*.

The appearance of circulating b5 is clearly associated with the appearance of b5-bearing lymphocytes (Figs. 1 *c* and *e*). However, the apparent lag between

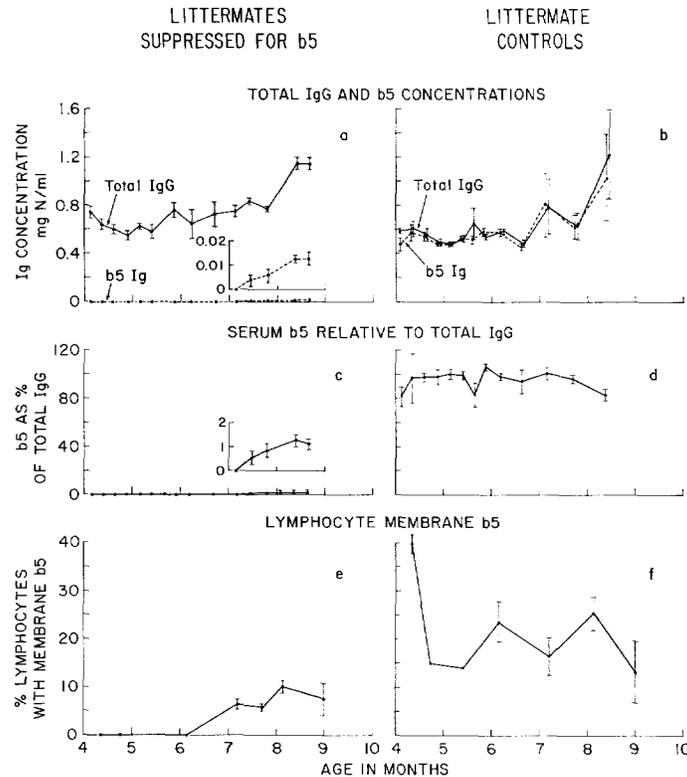


FIG. 1. Circulating b5 Ig levels and percent of peripheral blood lymphocytes with membrane b5 Ig in suppressed and normal littermates during escape from allotype suppression. Five suppressed littermates (K165-2, -4, -5, -6, -7) and two normal untreated littermates (K165-1, -3) were examined from age 4 to 10 mo. Panels *a* and *b* illustrate the relation of b5 and total IgG concentrations in serum. In panels *c* and *d* circulating b5 Ig is plotted as percent of total IgG = $\frac{\text{b5 mg N/ml}}{\text{IgG mg N/ml}} \times 100$. In panels *e* and *f* percent lymphocytes with membrane b5 = $\frac{\text{no. of PBL labeled with } ^{125}\text{I anti-b5}}{\text{total no. of PBL counted}} \times 100$. Points and bars represent the geometric mean \pm SEM. The insets in *a* and *c* show on an expanded scale the very small amounts of circulating b5 Ig produced after escape from suppression.

detection of lymphocytes with membrane b5 by ^{125}I autoradiography and circulating b5 by radial diffusion in gel may simply reflect the relative insensitivity of the latter method. Therefore serum samples from the crucial escape period were retested for the first detectable b5 by a hemagglutination inhibition (HAI) assay which was approximately 100 times more sensitive than radial diffusion in gel (capable of detecting $\sim 1 \mu\text{g/ml}$ of b5 immunoglobulin). In escaping rabbits' sera, b5 was detectable by HAI 2 wk earlier than by radial diffusion. Yet three of the five suppressed rabbits still had 4–7% of PBL with

detectable membrane b5 before even $\sim 1 \mu\text{g/ml}$ of circulating b5 was present by HAI. In no case was serum b5 detected before lymphocyte membrane b5.

During Recovery from Suppression, the Number of Lymphocytes with Membrane b5 Returned Toward Normal While the Serum b5 Levels Remained Depressed.—A striking feature of allotype suppression in the rabbit is that serum levels of the affected allotype remain depressed for long periods even after some synthesis has occurred. We found, however, that b5-bearing lymphocytes recovered toward normal levels more quickly. This trend was already demonstrable in litter K165 at 8½ mo of age (Fig. 1). While serum b5 levels were still 2% of the normal levels found in untreated littermates (Figs. 1 *c* and *d*), the percent of b5-bearing lymphocytes in peripheral blood was already about 40% of normal littermate levels (Figs. 1 *e* and *f*).

Fig. 2 provides an overview of our experiments and further illustrates the discrepancy between serum b5 levels and the proportion of lymphocytes with membrane b5 in rabbits recovering from allotype suppression. Here we compare serum b5 levels and percent of b5-bearing lymphocytes in peripheral blood in four litters at various stages of recovery from suppression. The range of values for normal rabbits is indicated to demonstrate the discrepancy between serum b5 relative to normal and b5-bearing lymphocytes relative to normal. Thus serum b5 levels remain chronically depressed and well below the normal range up to 23 mo of age (Fig. 2 upper panel). While the percent of b5-bearing lymphocytes varies over a wide range in normal b5 rabbits, it is clear that the percent of b5-bearing lymphocytes in recovering rabbits returned rapidly toward normal after escape from suppression (Fig. 2 lower panel). In suppressed rabbits over 9 mo of age (litters K326, H2, and H206), the percent of b5-bearing lymphocytes in peripheral blood became indistinguishable from that in normal b5 rabbits. Yet circulating b5 Ig levels in those rabbits remained clearly sub-normal, measuring less than 30% of normal even at 23 mo of age.

We considered the interesting possibility that the b5 light chains produced in escape and recovery from suppression may have been associated exclusively with IgM molecules (possibly reflecting inability to make the IgM to IgG “switch”) and thus underestimated in the radial diffusion assay because of the high molecular weight of IgM. However, immunoelectrophoresis of sera from a recovering rabbit (K326) making less than 5% of normal circulating b5 revealed b5 associated with an IgG arc. In addition, the low b5 levels in recovering rabbits were confirmed by the HAI assay which would not be affected by molecular size.

DISCUSSION

The mechanism by which perinatal exposure to appropriate anti-Ig antisera suppresses production of a specific class (14, 15), idiotype (16), or allotype (7–10, 12, 13) is not known. For chronic allotype suppression in the rabbit, we have demonstrated a complete deletion of b5-bearing lymphocytes from the

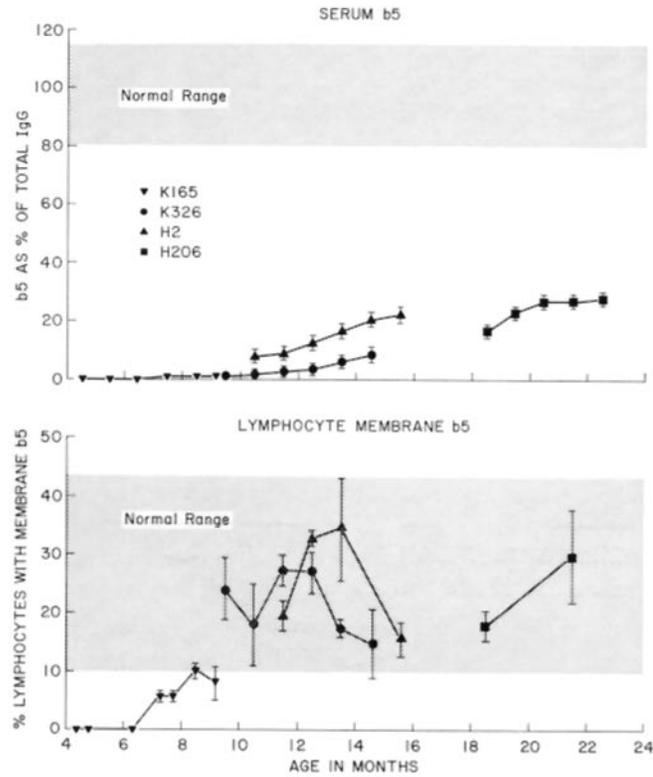


FIG. 2. The recovery of circulating b5 Ig and peripheral blood lymphocytes with membrane b5 Ig after escape from allotype suppression. b5 homozygous rabbits from four different litters were suppressed with anti-b5 antisera at birth and studied over the indicated age period. Five K165 littermates, two K326 littermates, and one each from litters H2 and H206 were examined. Each point and bar represents the geometric mean \pm SEM of all values obtained during any given month of age. $b5 \text{ as percent of total IgG} = \frac{b5 \text{ mg N/ml}}{\text{IgG mg N/ml}} \times 100$.

$$\text{Percent lymphocytes with membrane b5} = \frac{\text{no. of PBL labeled with } ^{125}\text{I anti-b5}}{\text{total no. of PBL counted}} \times 100.$$

The range of values found in normal b5 homozygous rabbits is indicated to illustrate the discrepancy between serum b5 levels which remain chronically depressed and the proportions of PBL with membrane b5 which become indistinguishable from normal.

peripheral blood during total suppression for b5.² Our observations strongly support the proposition that allotype suppression is mediated by elimination of b5-bearing small lymphocytes or their precursors. Recent identification of cells

² Since we wanted to study these valuable rabbits during recovery from suppression, they could not be sacrificed to establish a similar deletion of b5-bearing lymphocytes in other lymphoid organs.

bearing the paternal allotype in newborn b4b5 heterozygote rabbits in our laboratory³ further supports the view that the induction of allotypic suppression involves the interaction of anti-Ig antibodies with Ig-bearing precursor cells. Elimination of cells bearing the suppressed type Ig after treatment with anti-Ig antisera has also been documented in the case of Ig class suppression in chickens (14) and mice (15), but not in the case of allotype (12, 13) or idiotype (16) suppression in mice. Although the mechanism by which Ig-bearing precursor cells are eliminated and prevented from reemerging is not known, the chronicity of total allotype suppression in rabbits (up to 14 mo [19]) may be explained by some active suppressor in these rabbits capable of eliminating Ig-bearing precursor cells as they emerge from the stem cell pool. Such an active suppressor function has recently been suggested for thymus-derived cells in a variety of systems (28) and specifically for allotype suppression in (SJL × BALB/c) F₁ mice (29, 30).

Escape and recovery from allotype suppression provides a unique model for study of B cell development. Our studies demonstrate that in escape from suppression the appearance of secreted b5 Ig is chronologically linked to the appearance of b5-bearing lymphocytes. Our experiments strongly suggest that the differentiation of an Ig-secreting cell capable of producing b5 is dependent on the presence of a precursor lymphocyte with b5 Ig on its membrane. Since Ig-secreting cells generally make only one allotype (31) and Ig-bearing cells likewise generally express only one allotype on their membrane (4, 6), our observations suggest but do not prove that commitment to one of several allotypes represented in the genome may take place quite early in the process of B cell maturation. Although little data are available about the relation between restrictions on Ig on precursor cell membranes and restrictions on Ig secreted by more differentiated cells, the finding that the allotype of membrane and cytoplasmic Ig in single cells is always the same (32) suggests for single cells what our studies suggest for an *in vivo* cell population, i.e., that restriction to one allotype is operative at both the immunoglobulin-bearing cell stage and the antibody-secreting cell stage.

There are several possible interpretations of the finding of low serum levels of b5 and relatively more normal proportions of b5-bearing lymphocytes in rabbits recovering from allotype suppression. Suppression of b5 kappa-type light chains may have led to compensatory expansion of the pool of antigen-sensitive precursor cells bearing lambda-type light chains to cover the antigen specificities normally associated with b5 receptors. Thus the recovering b5-bearing cells may have been less frequently stimulated to go on to Ig-secreting cells because their restricted range of receptor specificities made encounter with appropriate antigen rare. Another possibility is that there is a block in B cell maturation that prevents normal b5-bearing precursor lymphocytes from

³ M. R. Harrison and R. G. Mage, unpublished observations.

going on to b5-secreting cells after appropriate stimulation. Such blocks in cyto-differentiation have been recently identified at several stages in B cell maturation (33, 34). Finally, the cellular machinery for production of b5 may be entirely intact, but the secretion of b5 inhibited by extrinsic regulatory factors such as suppressor cells (28-30) or lambda-type Ig molecules acting as feedback inhibitors (35, 36).

SUMMARY

The proportion of peripheral blood lymphocytes with cell surface b5 immunoglobulin (Ig) was compared with serum b5 Ig levels in allotype-suppressed and recovering b5 homozygous rabbits in order to establish the cellular defect in suppression and to determine the relation between lymphocyte membrane Ig and secreted Ig of the same allotype.

In rabbits fully suppressed for b5, the absence of circulating b5 Ig is reflected in a complete deletion of b5-bearing lymphocytes. During spontaneous escape from suppression, the appearance of b5-bearing lymphocytes precedes the appearance of detectable serum b5. During recovery from suppression b5-bearing lymphocytes recover rapidly toward normal levels, but circulating b5 levels remain chronically and disproportionately depressed.

We are grateful to Glendowyn O. Young-Cooper, Cornelius Alexander, and Rudolph R. Sullivan (Flow Laboratories Inc., Rockville, Md.) for excellent technical assistance.

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