ALLOTYPE SUPPRESSION IN THE RABBIT

I. THE ONTOGENY OF CELLS BEARING IMMUNOGLOBULIN OF PATERNAL ALLOTYPE AND THE FATE OF THESE CELLS AFTER TREATMENT WITH ANTIALLOTYPE ANTISERA

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Since Dray’s original description of allotype suppression in the rabbit (1), suppression of immunoglobulin (Ig)$^1$ allotypes in mice (2, 3), of Ig classes in chickens (4) and mice (5), and of Ig idiotypes in mice (6) has been documented. Nonetheless, the mechanism by which exposure to appropriate anti-Ig antisera suppresses production of a specific allotype, class, or idiotype is not known.

In the rabbit, the expression of $b$ locus allotypic determinants present on kappa-type light chains can be suppressed by exposing rabbits in utero or at birth to appropriate antiallotype antisera (allotype suppression) (1, 7-9). It is reasonable to hypothesize that an early event in the establishment of suppression is the interaction of the injected antibodies with specific Ig determinants present on the membranes of bone marrow-derived lymphocytes (B lymphocytes) or their precursors. However, in the rabbit an appropriate Ig-bearing “target” cell has not previously been demonstrated in the crucial perinatal period when antiallotype antisera are effective in inducing suppression, nor has the fate of such a target cell after interaction with suppressing antibodies been studied. Our previous studies demonstrating a complete deletion of $b5^b$-bearing lymphocytes from the peripheral blood of adult homozygous $b5^b$ rabbits suppressed for $b5$ (10) suggested that such Ig-bearing target cells may be eliminated during induction of allotype suppression.

The study of B cell ontogeny in most species is complicated by the presence of maternally derived Ig in the newborn. The study of an Ig marker not found in the mother but inherited from the father allows positive identification of B cells bearing endogenously produced Ig. Thus Ig allotypes provide a powerful tool for study of B cell ontogeny in the heterozygous offspring of parents of known genotype. In the present studies we made use of rabbits heterozygous at the $b$ locus to document the cellular events surrounding induction of suppression in the neonate.

Materials and Methods

Animals.—Rabbits used in these experiments were of known parentage and allotype from closed colonies bred at the N.I.H. Sera and antisera were characterized as previously described (11).

$^1$ Abbreviations used in this paper: FL, fluoresceinated; HAI, hemagglutination inhibition; Ig, immunoglobulin; MEM, minimal essential medium.
Quantitation of Ig Concentrations in Sera.—Sera were assayed for total concentrations of IgG, b4, and b5 by radial diffusion in gels and hemagglutination inhibition (HA1) as previously described (10).

Cell Suspensions from Perinatal Rabbits.—Lymphocytes from blood, spleen, appendix, and bone marrow were prepared by a modification of methods previously described (10). Blood was collected in heparin by decapitation and pooled for each litter; erythrocytes were sedimented with methyl cellulose-Hypaque and lymphocytes isolated over Ficoll-Hypaque. Splenocytes were obtained by teasing spleens in Petri dishes and filtering through cotton gauze to remove debris. Lymph node lymphocytes were prepared in the same manner. Appendiceal cells were prepared by opening the excised appendix longitudinally, washing thoroughly, scraping cells off the mucosal surface, and filtering through gauze. Bone marrow cells were obtained by washing out amputated femurs with medium and filtering through gauze. Pooled cell suspensions were then washed in minimal essential medium (MEM), erythrocytes lysed with NH4Cl, and the cells washed again and resuspended in MEM supplemented with heparin and sodium azide as described (10).

Preparation of Fluorescein-Conjugated Specific Antiallotype Antisera.—Specific antisera were produced by immunization of b4b4, b5b5, and b4b5 rabbits with purified b5, b4, and b9 IgG, respectively (11). IgG fractions of the b4 anti-b5, b5 anti-b4, and b4b5 anti-b9 were prepared by precipitation with 18% Na2SO4 and chromatography on DEAE-cellulose equilibrated with 0.0175 M sodium phosphate buffer, pH 6.9. The purified IgG was fluoresceinated by the dialysis method (12). These fluoresceinated (F1) antibodies were specific and non-cross-reacting when tested for precipitation by double diffusion in agar gels and when used to label lymphocyte preparations from homozygous rabbits of known allotype.

Detection of Membrane-Associated Ig Allotype with Fluorescent Antiallotype Antibodies.—1–10 million cells suspended in 0.2 ml of MEM containing heparin and sodium azide (1 mg/ml) were incubated with 0.1–0.2 mg (20–40 µl) of fluoresceinated antiallotype antibody at 4°C for 30 min, washed twice in the same azide containing medium, and examined using a standard optical system for fluorescence microscopy as described (10). Since labeling of viable lymphocytes was carried out at 4°C in the presence of azide, only membrane-associated Ig determinants were detected, and most cells revealed a patchy distribution of label.

RESULTS

Ontogeny of B Lymphocytes Bearing Endogenously Synthesized Surface Ig.—The results of staining lymphocytes from peripheral blood, spleen, appendix, and bone marrow of neonatal rabbits for both paternally and maternally inherited allotypes are presented in Table I. Cells bearing the b locus allotype inherited from the father were present in substantial numbers in the peripheral blood and spleen of newborn rabbits from two separate litters. These cells were also present in the appendix at birth, and were demonstrable in peripheral blood, spleen, and appendix through the 1st wk of life. Bone marrow contained cells bearing paternal allotype in small numbers when first examined at 2 days of age.

In contrast to the very early appearance of endogenously synthesized paternal allotype on cell membranes, circulating Ig of paternal allotype was not present in detectable amounts until approximately 2 wk of age. Thus, in postnatal ontogeny, endogenously synthesized Ig of paternal allotype appears first on lymphocyte membranes and only later appears as secreted Ig.

Elimination of Lymphocyte Membrane b5 Ig after In Vivo Treatment with Anti-b5 Antisera.—Since a potential target cell for allotype suppression could
TABLE I
Ontogeny of Rabbit B Cells: Appearance of Cells Bearing Paternally* and Maternally
Inherited Ig Allotypes

| Exp. no. | Age of litter | Parental source of b4 and b5 allotypes | Cells stained with fluoresceinated | Percent of fluorescent-positive cells in each organ | Serum Ig of paternal allotype§ |
|----------|---------------|----------------------------------------|----------------------------------|-----------------------------------------------|--------------------------------|
|          |               | b5 anti-b4                             | %                                | %                             | %                             | µg/ml                          |
| 1 Newborn| Paternal b4    | 5.5                                    | 11.9                             | N.D.                           | N.D.                           | <0.001                         |
|          | Maternal b5    | 8.9                                    | 5.4                              | N.D.                           | N.D.                           |                                |
| 2 Newborn| Paternal b4    | 12.9                                   | 10.7                             | 4.7                            | N.D.                           | <0.001                         |
|          | Maternal b5    | 19.8                                   | 6.1                              | 2.9                            | N.D.                           |                                |
| 3 2 Days | Paternal b4    | 16.4                                   | 7.1                              | N.D.                           | N.D.                           | <0.001                         |
|          | Paternal b5    | 6.7                                    | 3.4                              | N.D.                           | N.D.                           |                                |
| 4 4 Days | Paternal b4    | 6.6                                    | 19.6                             | N.D.                           | 1.8                            | <0.001                         |
|          | Paternal b5    | 7.9                                    | 13.1                             | N.D.                           | 1.6                            |                                |
| 5 7 Days | Paternal b4    | 21.5                                   | 9.8                              | 11.5                           | N.D.                           | <0.001                         |
|          | Paternal b5    | 14.2                                   | 6.3                              | 9.0                            | N.D.                           |                                |
| 6 10 Wk  | Paternal b4    | 44.0                                   | 43.3                             | 6.1                            | 4.9                            | 0.325                          |
|          | Paternal b5    | 31.0                                   | 35.0                             | 3.1                            | 3.6                            |                                |

* Ig of paternal allotype must be endogenously synthesized; Ig of maternal allotype may be passively acquired from the mother.
† At least 1,000 cells were counted in each preparation. N.D., not done.
§ Paternal allotype is not detectable by a sensitive hemagglutination inhibition assay capable of detecting 0.001 mg Ig/ml until ~2 wk of age.

be identified, we examined the fate of this b5 Ig-bearing cell after treatment of new born b4b5 rabbits with b9 anti-b5 antisera. Peripheral blood, spleen, and bone marrow from treated and control rabbits were examined for (a) cells bearing maternal b4 Ig by direct staining with Fl b5 anti-b4, (b) cells bearing paternal b5 Ig by direct staining with Fl b4 anti-b5, and (c) cells whose b5 determinants might have been masked by the injected b9 anti-b5 antibodies by indirect staining with Fl b4b5 anti-b9.

The results of two experiments are reported in Table II. In exp. 1, a litter of new born b4b5 rabbits (paternal b5) was treated with b9 anti-b5 on the 1st and 2nd days of life while another litter born the same day was left untreated. 48 h after the last injection, at 4 days of age, the litters were sacrificed and peripheral blood, spleen, and bone marrow from each group were pooled and examined. Cells from the organs of the treated group no longer had detectable membrane b5 Ig, while the percentages of cells with maternal b4-type Ig were comparable to those of the untreated litter (Table II, exp. 1). In order to further
| Treatment of neonatal b4b5 rabbits* | Organs examined | Percent fluorescent-positive cells1 |
|-----------------------------------|-----------------|-----------------------------------|
|                                   | b4 Cells (PI b5 anti-b4) | b5 Cells (PI b4 anti-b5) | Cells coated with b9 (PI b4b5 anti-b9) |
| b9 anti-b5 48 h after last injection | Blood           | 5.1 < 0.2 < 0.2                   |
| 3 ml i.p. at birth (Age < 4 days)  | Spleen          | 22.2 < 0.2 < 0.2                  |
| 5 ml i.p. on day 1                 | Bone marrow     | 1.5 < 0.2 < 0.2                   |
| Newborn b4b5 rabbits              | Blood           | 6.6 7.9 < 0.2                     |
|                                   | Spleen          | 19.6 13.1 < 0.2                   |
|                                   | Bone marrow     | 1.8 1.6 < 0.2                     |
| No treatment Same                 | Blood           | 10.6 < 0.2 < 0.2                  |
|                                   | Spleen          | 7.0 < 0.2 < 0.2                   |
|                                   | Bone marrow     | 2.5 < 0.2 < 0.2                   |
|                                  | Blood           | 16.4 6.7 < 0.2                    |
| b9 anti-b5 24 h later             | Spleen          | 7.1 3.4 < 0.2                     |
| 5 ml i.p. at birth (Age < 2 days) | Bone marrow     | 2.0 1.4 < 0.2                     |
| Newborn b4b5 rabbits              | Blood           |                                   |
| No treatment Same                 | Spleen          |                                   |
|                                   | Bone marrow     |                                   |

* Each group consisted of three to five neonatal rabbits. Cells from each organ were pooled to provide enough cells for examination.

† At least 500 cells were counted in each preparation. In animals treated with anti-b5 no b5-bearing cells were detected. b9 anti-b5 antibodies were not detectable on the surface of these cells by sensitive fluorescent staining with b4b5 anti-b9 antiserum (see Table III).
define the kinetics of this apparent elimination of b5-bearing target cells by anti-b5 antiserum, a second experiment was done in which newborns were treated with a single injection of b9 anti-b5 within the 1st 24 h of life and their organs examined 24 h later, i.e., <48 h after birth. Again cells bearing paternal b5 Ig were not detectable in animals given b9 anti-b5 24 h earlier (Table II, exp. 2). In both experiments, cells bearing the nonsuppressed b4 Ig were unaffected by anti-b5 treatment. In addition, staining with Fl b4b5 anti-b9 did not reveal any cells with the injected b9 anti-b5 on their membrane. Preliminary studies had established that Fl b4b5 anti-b9 could detect b9 anti-b5 antibodies coated in vitro onto peripheral blood lymphocytes from b4b5 rabbits (Table III). Indeed, indirect staining with b4b5 anti-b9 was even more sensitive than direct staining with b4 anti-b5, possibly due to the amplification effect of two-step staining. We conclude from these experiments that lymphocyte membrane b5 Ig was eliminated from the peripheral blood, spleen, and bone marrow within 24 h of injection of b9 anti-b5 antiserum and was not merely masked by the injected antiserum.

**b4-Bearing Cells and b5-Bearing Cells in b4b5 Rabbits Suppressed for b5.**—In order to document the fate of b5-bearing cells during complete suppression and in escape from suppression we examined the peripheral blood, spleen, lymph nodes, appendix, and bone marrow of heterozygous b4b5 rabbits suppressed for b5 at birth for both b4-bearing cells and b5-bearing cells. In each of three separate experiments, the serum level of b4 and b5 Ig as well as the proportion of b4- and b5-bearing lymphocytes in each organ of a suppressed rabbit was compared with that of an untreated littermate.

The results are presented in Table IV. Exp. 1 demonstrates that in a fully suppressed rabbit at 4 wk of age there were no cells bearing detectable paternal b5 allotype in the peripheral blood, spleen, lymph nodes, appendix, or bone marrow. In exps. 2 and 3 we examined suppressed and normal rabbits at 10

| Preincubation* | Fluorescent staining reagent | Percent fluorescent-positive cells |
|----------------|-------------------------------|-----------------------------------|
| MEM alone      | b4 anti-b5                    | 24.8                              |
| MEM alone      | b4b5 anti-b9                  | <0.2                              |
| b9 anti-b5 antiserum | b4b5 anti-b9                  | 53.1                              |
| b4 anti-b5 antiserum | b4b5 anti-b9                  | <0.2                              |
| b9 normal serum | b4b5 anti-b9                  | <0.2                              |

* Peripheral blood cells from b4b5 donors were suspended in MEM containing heparin, azide, and 10% fetal calf serum (FCS). For preincubation, 10% b9 anti-b5, 10% b4 anti-b5, or 10% normal b9 serum was added for 30 min at 4°C. Cells were then washed twice through FCS, resuspended, and stained with fluoresceinated reagents.
| Exp. no. | b<sup>4</sup>b<sup>4</sup> Rabbits | Age | Serum Ig allotype concentration | Cells stained with | Percent fluorescent-positive cells in each organ |
|---------|---------------------------------|-----|--------------------------------|-------------------|-----------------------------------------------|
|         |                                 |     | mg/ml                           |                   | Peripheral blood | Spleen | Lymph node | Appendix | Bone marrow |
| 1       | Suppressed for b<sub>5</sub> at birth | 4   | 0.450 < 0.001*                    | Fl b<sub>5</sub> anti-b<sub>4</sub> | 31.8 | 9.5 | 24.0 | 4.7 | 1.2 |
|         |                                 |     |                                 | Fl b<sub>4</sub> anti-b<sub>5</sub> | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
|         | Normal untreated littermate     | 4   | 0.437 0.275                     | Fl b<sub>5</sub> anti-b<sub>4</sub> | 13.3 | 5.2 | 17.4 | 4.3 | 1.0 |
|         |                                 |     |                                 | Fl b<sub>4</sub> anti-b<sub>5</sub> | 7.9 | 3.8 | 12.9 | 2.6 | 0.8 |
| 2       | Suppressed for b<sub>5</sub> at birth | 10  | 1.562 < 0.001*                    | Fl b<sub>5</sub> anti-b<sub>4</sub> | 61.8 | 74.4 | 51.0 | 3.7 | 14.7 |
|         |                                 |     |                                 | Fl b<sub>4</sub> anti-b<sub>5</sub> | 0.6 | <0.2 | <0.2 | <0.2 | 1.9 |
|         | Normal untreated littermate     | 10  | 0.937 0.500                     | Fl b<sub>5</sub> anti-b<sub>4</sub> | 58.2 | 80.9 | 40.2 | 2.6 | 10.9 |
|         |                                 |     |                                 | Fl b<sub>4</sub> anti-b<sub>5</sub> | 35.1 | 35.7 | 20.2 | 3.0 | 5.7 |
| 3       | Suppressed for b<sub>5</sub> at birth | 11  | 2.750 < 0.001*                    | Fl b<sub>5</sub> anti-b<sub>4</sub> | 70.1 | 42.9 | 13.6 | 3.6 | 6.2 |
|         |                                 |     |                                 | Fl b<sub>4</sub> anti-b<sub>5</sub> | 1.0 | <0.2 | <0.2 | <0.2 | 0.3 |
|         | Normal untreated littermate     | 11  | 1.500 0.325                     | Fl b<sub>5</sub> anti-b<sub>4</sub> | 44.0 | 43.3 | 29.6 | 6.1 | 4.9 |
|         |                                 |     |                                 | Fl b<sub>4</sub> anti-b<sub>5</sub> | 31.0 | 35.0 | 14.3 | 3.1 | 3.6 |

* No b<sub>5</sub> was detectable by radial diffusion in gels. By an HAI assay < 100 times more sensitive (capable of detecting < 1 µg/ml), no serum b<sub>5</sub> was detectable in exps. 1 and 2 and only a trace was detectable in exp. 3.
and 11 wk of age. In both experiments a small but definite number of b5-bearing cells had appeared in bone marrow and peripheral blood, providing the first indication that the suppressed animals were about to escape from total allotype suppression. Although no circulating b5 Ig could be detected by standard radial diffusion in gels, tests of the sera by a sensitive HAI assay capable of detecting 1 μg/ml revealed the first traces of circulating b5 Ig in one of these rabbits. At this early stage no b5-bearing cells could be found in spleen, appendix, or lymph nodes.

In all three experiments the number of cells bearing the nonsuppressed b4 allele was generally increased above normal in the b5-suppressed rabbits. This was especially evident in peripheral blood and spleen. Thus in suppressed heterozygotes there was a compensatory increase in expression of the nonsuppressed b locus allele on lymphocyte membranes analogous to that found in serum. (In Table IV, compensatory production of serum b4 Ig is evident at 10 and 11 wk of age but at 4 wk residual injected and maternally derived b4 Ig is still present.) Allelic compensation, previously documented for secreted Ig (1, 9), can now be extended to the expression of Ig allotypes on lymphocyte membranes.

DISCUSSION

In b4b5 heterozygous offspring of parents homozygous at the b locus Ig controlled by genes inherited from the dam and sire can be identified both on lymphocyte membranes and in the serum (10). The ability to distinguish Ig of paternal allotype in neonatal b4b5 rabbits has allowed us to circumvent the problem of passively acquired maternal Ig, to document the postnatal ontogeny of B cells bearing endogenously synthesized Ig of paternal type, and to explore the fate of these cells after exposure to antiallotype antibodies during the induction of allotype suppression.

In normal b4b5 rabbits, cells bearing endogenously synthesized Ig of paternal allotype were present in peripheral blood (6–12% fluorescent-positive cells), spleen (11–12%), and appendix (~5%) shortly after birth (Table I). Thus a potential target cell for suppressing antiallotype antibodies, the cell bearing Ig of paternal allotype, was present during the crucial perinatal period when antiallotype antibodies can initiate chronic allotype suppression.

Within 24 h of injection of b9 anti-b5 antisera into newborn b4b5 rabbits, cells bearing paternal b5 Ig were no longer demonstrable in peripheral blood, spleen, or bone marrow. Cells bearing maternal b4 Ig were not affected. Moreover, the suppressing b9 anti-b5 antibodies were not detectable on the surface of cells in these organs by sensitive fluorescent staining with b4b5 anti-b9 antiserum (Table II). Since this b4b5 anti-b9 antiserum was capable of detecting b9 Ig specifically coated on b4b5 cells in vitro (Table III), the absence of detectable b9 marker on cell membranes demonstrated that the injected b9 anti-b5 antibodies were not simply masking the b5 determinants on the membranes of
the target cells. Finally, examination of suppressed and normal b5 rabbits at 4, 10, and 11 wk of age revealed that membrane Ig of paternal b5 allotype was completely deleted from peripheral blood, spleen, lymph node, appendix, and bone marrow for the duration of total allotype suppression, and reappeared first in bone marrow and peripheral blood at the time of escape from suppression (Table IV). We concluded from these studies that during the induction of total allotype suppression there was rapid and complete elimination of membrane-associated Ig of the suppressed allotype after interaction with antiallotype antibody.

These studies on the induction of allotype suppression in neonatal rabbits establish that the b5-bearing target cells were either eliminated or their membrane Ig was modulated from the surface (13) and not replaced with the original light chain form. The in vitro consequences of interaction of anti-Ig antibodies with membrane Ig have been well documented. Anti-Ig antisera produce blastogenic transformation in vitro (14). At the microscopic level rapid migration of the complexes within the membrane to form patches or caps is followed by endocytosis, pinocytosis, or sloughing of the aggregated complexes (15–17). Unfortunately, the subsequent fate of such cells in vivo is not known. Since it is necessary to maintain an excess of circulating antiallotype antibody in the milieu during establishment of chronic allotype suppression, any Ig of the same suppressed allotype that reappeared on the cell surface would immediately encounter additional antiallotype antibody. Such cells may “re-differentiate” and produce another light chain type or may be eliminated by continued assault upon the membrane Ig by anti-Ig antibodies. Although direct cytotoxicity of antiallotype antisera for allotype-bearing lymphocytes in the presence of complement is generally not demonstrable in vitro (18), it is possible that the target cells are cleared by reticuloendothelial cells, killed by nonspecific cytotoxic cells, or that they die out after transformation into blast cells (“abortive differentiation”). From recent work in our laboratory we now know that cells from neonatal rabbits are capable of blastogenic responses to antiallotypic antisera as well as mitogens. However, simply causing blast transformation is not necessarily sufficient for the establishment and chronic maintenance of a suppressed state (19–21).

The mechanism by which Ig-bearing precursor cells are prevented from reemerging for weeks to years in chronic allotype suppression may not be the same as that operating in the initial induction of suppression. The chronicity of allotype suppression in rabbits may be explained by some active suppressor function that eliminates Ig-bearing cells as they emerge from the stem cell pool. Such an active suppressor function has recently been demonstrated for thymus-derived lymphocytes in a variety of systems (22) and specifically for allotype suppression in (SJL × BALB/c)F1 mice (23, 24). The role of thymus-
dependent cells in the maintenance of chronic allotype suppression in the rabbit is under investigation, but the rapid elimination of allotype-bearing cells by antiallotype antisera makes it unlikely that sensitized suppressor T cells play a role in the initial induction of allotype suppression in neonatal rabbits.

Neonatal ontogeny and escape from allotype suppression also provided an opportunity to study B cell development and the relationship between lymphocyte membrane Ig and circulating Ig of the same allotype. Although during postnatal ontogeny, cells bearing endogenously synthesized Ig of paternal allotype were present from birth, circulating b5 Ig was not detectable until \( \sim 2 \) wk of age. Similarly, in the “artificial ontogeny” of escape from allotype suppression, the appearance of b5-bearing lymphocytes in bone marrow and peripheral blood heralded the appearance of circulating b5 Ig. These studies of suppressed \( b^b_4 \) heterozygotes confirm and extend our previous studies of peripheral blood lymphocytes in suppressed homozygous rabbits (10). In each instance the appearance of secreted b5 Ig has been chronologically linked to the prior appearance of b5-bearing lymphocytes, strongly suggesting 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. The present studies also established a link between the number of b4-bearing lymphocytes and the amount of b4 secreted in \( b^b_4 \) rabbits suppressed for b5, i.e., compensatory production of the nonsuppressed allelic allotype in serum (1, 9) appears to reflect increased numbers of b4 Ig-bearing precursor cells. All these observations suggest that commitment to one of several light chains represented in the genome may take place quite early in the process of B cell maturation, such that the allotype of the Ig receptors on B cells is also the allotype of the eventual secreted Ig product.

**SUMMARY**

The ability to distinguish immunoglobulin (Ig) of paternal allotype both on lymphocyte membranes and in the serum of neonatal \( b^b_4 \) heterozygous rabbits has allowed us to study the postnatal ontogeny of cells bearing endogenously synthesized Ig that could not have been derived from the mother. In normal \( b^b_4 \) rabbits, such endogenously synthesized Ig of paternal allotype is present on the membranes of bone marrow-derived (B) lymphocytes from birth, but does not appear as detectable circulating Ig until \( \sim 2 \) wk of age.

In the neonate, cells bearing the paternal allotype are potential targets for the induction of chronic allotype suppression. Within 24 h of exposure to anti-allotype antisera in vivo, Ig of the suppressed paternal allotype is no longer detectable on the surface of neonatal lymphoid cells. Further, this lymphocyte membrane Ig is eliminated and not simply masked by the suppressing antibodies. Finally, cells bearing the suppressed allotype are deleted from all lymphoid organs for the duration of total allotype suppression, and reappear first in bone marrow and peripheral blood at the time of spontaneous escape from suppression.
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