CHANGES IN SUPPRESSOR MECHANISMS DURING POSTNATAL DEVELOPMENT IN MICE*

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The functioning of T lymphocytes in regulation of antibody responses has been recognized as both helping and suppressing the responsive bone marrow-derived lymphocyte (B cell)[1] (reviewed in 1). With regard to the termination of the response, many suppressor systems have been described, some nonspecific, spontaneous (2–4), or induced (5–7), some specific in their induction and nonspecific in their activity (8–10), or some specific both in their induction and in their effector phases (11–13).

In a previous report, we described the activity of specific suppressor cells among normal splenic lymphocytes cultured in the presence of specifically primed spleen cells (11). When these two cell populations (normal and sheep erythrocyte [SRBC] immune) were mixed and challenged with SRBC, both the primary response of the normal cells and the secondary response of the immune cells were suppressed. The suppression is specific in that the primary response to trinitrophenylated chicken erythrocytes (TNP-CRBC) is not suppressed in the same cultures that show suppressed SRBC responses. That the suppressor cells are mature thymus-derived lymphocytes (T cells) is suggested by their nonadherence to nylon wool (11), their presence in lymph node and cortisone-resistant thymus cell populations, their lower sensitivity to anti-Thy.1 and C, and their relative absence from the normal thymus (14).[2] The mechanism of this suppression may involve specific in vitro induction of specific suppressor cells by primed spleen cells.

The present study was undertaken to determine the existence of this form of suppression in younger animals and to relate it to the neonatal nonspecific primary response suppression (15). The data suggest that the numbers of suppressor cells assayed in the above system are reduced in spleens of 3-wk-old animals, whereas they are present at adult levels or even higher in 1-wk-old animals. This seems an unorthodox pattern of development for a single cell subpopulation. The data support the notion that the 1-wk suppressor cell represents a different subpopulation from the adult suppressor cell previously described.

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cell; B cell, bone marrow-derived lymphocyte; Con A, concanavalin A; HBSS, Hanks' balanced salt solution; Ig^+, positive for surface immunoglobulin; LPS, bacterial lipopolysaccharide; PFC, plaque-forming cell; PHA, phytohemagglutinin; SRBC, sheep erythrocytes; T cell, thymus-derived lymphocyte; TNP, trinitrophenyl hapten; TNP-CRBC, trinitrophenylated chicken erythrocytes; TNP-HRBC, trinitrophenylated horse erythrocytes.

[2] C. E. Calkins, F.-W. Shen, T. Stanton, and O. Stutman. Manuscript in preparation.

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Materials and Methods

Animals. CBA/H from our breeding colony and BDF_1 (C57BL/6xDBA/2) mice either from The Jackson Laboratory (Bar Harbor, Me.) or bred from Jackson stocks in our facilities were used. Within each experiment, all mice used were the same sex and strain.

Cell Preparation. Spleen and thymus tissues were gently teased in Hanks' balanced salt solutions (HBSS) into suspension. Nylon wool nonadherent cells were prepared as described by Julius et al. (16) by passage of the spleen cell suspension through syringes packed with washed nylon wool after a 45-min incubation at 35°C on the wool column. Cells collected in the first 50 ml effluent were considered nonadherent and represented between 15 and 30% of the starting cell numbers. An extra 50 ml of HBSS was then washed through the column and discarded before the removal of the adherent cells by teasing and shaking the wool vigorously. Some cell populations were depleted of macrophages by passage through Sephadex G-10 columns (Pharmacia Fine Chemicals, Piscataway, N. J.; 17) or by adherence to glass beads (18).

Cell Culture. Primary and secondary antibody responses were generated in culture as described by Mishell and Dutton (19). SRBC and TNP-CRBC were used as antigens. The preparation of TNP-CRBC has been described elsewhere (20), as have the controls for the effect of increased cell numbers on these responses (11). The procedure of mitogen stimulation of proliferation was done as described previously (21). Briefly, 1.25×10^5 cells were cultured in 0.075 ml RPMI 8% fetal calf serum with 0.25 μl phytohemagglutinin (PHA; Difco Laboratories, Detroit, Mich.), 0.25 μg bacterial lipopolysaccharide (LPS; derived from *Escherichia coli* strain 0 128:B12, Difco Laboratories) or 0.25 μg concanavalin A (Con A; ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) for 2 days before the addition of 1 μCi [^{3}H]thymidine. 16–20 h after [^{3}H]thymidine addition to the cultures, they were harvested with a multiple sample harvester and counted by a Packard B scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Experimental Protocol. As reported previously (11), immune spleen cells from adult mice primed 8 days earlier with SRBC and pertussis were put into Mishell and Dutton-type cultures with normal spleen cells from adult (greater than 10 wk) or young (3–21 days) animals. Duplicate cultures were assayed for direct and indirect plaque-forming cells (PFC) on days 4 and 5 by using a slide modification (19) of the Jerne plaque assay. Anti-TNP-PFC were assayed on trinitrophenylated horse erythrocyte (TNP-HRBC; 20) slides.

Antiserum Treatment. Mass cytolysis was accomplished with the incubation of 5×10^7 spleen cells/ml of 1:40 dilution of anti-Thy.1.2 antiserum (kindly donated by Dr. F.-W. Shen or prepared as described previously [22]). These were diluted, centrifuged, and incubated either in 1:20 selected rabbit complement or 1:5 guinea pig complement (1 ml complement dilution/5×10^7 spleen cells) for 45 min at 37°C.

Sensitivity to anti-Thy.1.2 antiserum was determined in microtiter wells, each containing 0.025 ml of antiserum or normal mouse serum dilution, 0.025 ml of lymphocytes (5×10^6 cell/ml) and 0.025 ml of rabbit serum (1:20) or guinea pig serum (1:5). Cytotoxicity was determined by vital dye exclusion.

Mixed Antiglobulin Rosette Assay. Lymphocytes were incubated in rabbit anti-mouse total globulins (1:10; Microbiological Associates, Bethesda, Md.) for 30 min on ice, as described previously (23), washed and then incubated with goat anti-rabbit IgG (1:10; GIBCO, Grand Island, N. Y.) for 30 min on ice. After washing, these lymphocytes were spun to a pellet with SRBC which had been sensitized with rabbit anti-SRBC (1:100; GIBCO). Surface Ig positive (Ig^+) cells were counted as live rosettes per total number of live cells.

Results

Ontogeny of the Normal Cells Able to Suppress Primed Responses In Vitro. When 10^7 normal spleen cells are added to cultures containing 4×10^6 immune spleen cells, suppression of both the normal primary and the primed secondary responses occurs (11). To determine the age dependency of this effect, 10^7 spleen cells from normal 3 day, 1 wk, 3 wk, or adult (10–20 wk) mice were added to the immune spleen cell cultures. As shown in Table I, spleens from mice less than 1 wk old have suppressor capacities comparable to those of

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TABLE I
Suppression of SRBC-Primed Responses by Normal Spleen Cells of Different Aged Mice

Donor age‡	Percent of control ± SE*			
	IgM		IgG	
	Day 4	Day 5	Day 4	Day 5
Adult§	20 ± 3	9 ± 4	49 ± 10	16 ± 4
3 wk	57 ± 17	65 ± 18	81 ± 18	50 ± 5
1 wk	14 ± 8	6 ± 5	59 ± 18	29 ± 9

* Controls were cultures of 4×10^6 adult immune spleen cells and SRBC to which no other cells were added. Data represent averaged PFC/culture of five separate experiments.

‡ 10^7 normal spleen cells from BDF_1 mice of different ages were added with SRBC to cultures of 4×10^6 adult immune spleen cells.

§ Normal adult spleen cells cultured separately (10^7 cells/culture) with SRBC generated an average primary response of 950 PFC/culture in these experiments. The primary responses are also being suppressed in the combination cultures. If this additional suppression is taken into account in the calculations of percent of control (control then being the response of 4×10^6 immune cell cultures plus the response of 10^7 normal cell cultures), the percent of control values is slightly lower when adult cells are the donor cells. There is no change when 3-wk or 1-wk cells are the donor cells as they do not contribute large numbers of responding cells. The 3-wk spleen cell response averaged 76 PFC/culture when cultured alone with SRBC. No PFC above background were detectable in similar cultures of 1-wk spleen cells.

adult mice. The 3-wk-old spleen cells are generally less active suppressors than either 1-wk or adult animals. The data presented in Table I represent both direct and indirect PFC responses in cultures of immune cells and normal suppressor cells assayed 5 days after initiation of the culture. Some variability in the amount of suppression is seen, particularly with 3-wk spleen cells, probably reflecting differences in rates of maturation of different litters. Similar relative degrees of suppression to those seen in Table I obtain when only 5×10^6 normal suppressor cells were added to the immune cell cultures.

Qualitative Differences in Plaques from the Various Suppressed Cultures. In the indirect plaque assay of immune cell cultures challenged with SRBC, in addition to the large plaques which are regarded as antibody-forming cells (AFC), there are many small, indistinct spots in the SRBC sheet. These are seen in the indirect assays of immune cell cultures and of cultures containing both immune cells and adult or 3-wk normal cells. These are absent from the direct assays of these same cultures and from indirect assays of cultures containing immune cells and 1-wk normal spleen cells. This observation suggests some difference in the mechanisms of suppression of the 1-wk and the older spleen cells (see Discussion).

Specificity of 1-wk Suppressor Cells. Cultures of SRBC immune spleen cells immunized with a noncross-reacting antigen (TNP-CRBC) in addition to SRBC exhibit suppressed responses to both antigens when spleen cells from the 1-wk donors are added to the cultures. This is demonstrated in Table II when 5×10^6 spleen cells from 1-wk animals are added to cultures of 4×10^6 adult

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TABLE II
Nonspecific Suppression by 1-Wk Spleen Cells

Cells added*	Day 4 direct PFC/culture	
	Anti-SRBC	Anti-TNP
	% control‡	
None	1,450 (100)	620 (100)
1-wk spleen	30 (1)	0 (0)

* Test cells (5×10^6 cells) were added with SRBC and TNP-CRBC to cultures of 4×10^6 SRBC immune adult spleen cells.

‡ The control response, used for the calculation of percents, was that of 4×10^6 SRBC immune adult spleen cell cultures challenged in vitro with both SRBC and TNP-CRBC and is represented in line 1 of this table.

SRBC primed spleen cells with SRBC and TNP-CRBC. The secondary response to SRBC and the primary response to TNP are both suppressed. In similar experiments (11) with the addition of normal adult spleen cells to the SRBC primed cells, only the SRBC response is suppressed. These data suggest that, unlike the adult suppressor cells, the 1-wk suppressors are nonspecific.

Activity of 1-wk Suppressor Cells in the Absence of Immune Cells. To establish whether the 1-wk cells require the presence of immune cells to become active suppressor cells (11), 5×10^6 1-wk spleen cells were added to cultures of 10^7 normal adult spleen cells and immunized with SRBC. These responses are compared with those of similar cultures to which 5×10^6 extra adult cells were added (Table III). The 1-wk spleen cells suppressed the normal primary response by 70%, whereas spleen cells from 3- or 5-wk-old mice were not significantly more suppressive than the same number of adult cells. The relatively small degree of suppression seen with the addition of adult cells is most likely due to changed kinetics of the primary response with the addition of 50% more responsive cells. The active suppressor cells from the 1-wk spleen population are found in the nonadherent fraction from nylon wool separations. The nylon adherent population from the 1-wk spleen contains no detectable suppressor cells.

Characteristics of the 1-wk Suppressor Cell. To test whether the relatively increased numbers of immature T cells (22) in the 1-wk spleens led to the increase in suppressive activity, 1-wk thymocytes were added to the immune cell cultures. As demonstrated in Table IV, 10^7 1-wk thymocytes added to 4×10^6 immune spleen cell cultures suppressed little if at all. Half as many 1-wk spleen cells were suppressive in parallel cultures. The enhancement seen in the day 5 responses of the cultures to which thymocytes were added occurred only in one experiment. Although similar observations have been made in cultures to which adult thymocytes were added (14 and fn. 2), these phenomena also occurred in only a few cultures.

As in the suppression of the primary response, the 1-wk cell responsible for the suppression of the secondary response is found in the nonadherent fraction of spleen cells passed through nylon wool columns (Table V). This cell is also depleted, albeit to varying extents, in the nylon adherent cell fraction, whereas

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TABLE III
Suppression of Adult Primary Antibody Response to SRBC by Lymphocytes of Young Mice

Donor age*	Percent suppression by spleen cell fractions ± SE‡ (number of replicate experiments)		
	Whole	Nonadherent	Adherent
wk			
1	70 ± 12 (9)	89 ± 1 (3)	4 ± 4 (2)
3	47 ± 17 (5)	ND§	ND
5	46 (1)	ND	ND
10	35 ± 10 (11)	ND	ND

* 5×10^6 cells added with SRBC to cultures of 10^7 normal spleen cells. The responses of these normal spleen cell cultures with no cells added were used as the 100% level for calculating percent suppression.
‡ These numbers represent averages of individual experiments ± standard errors, with the number of experiments in parentheses.
§ Not determined.

TABLE IV
Effect of 1-Wk Spleen and Thymus Cells on Secondary in Vitro Antibody Responses

Normal cells added*	Anti-SRBC PFC/culture (% control)‡			
	IgM		IgG	
	Day 4	Day 5	Day 4	Day 5
None	2,738 (100)	719 (100)	15,862 (100)	4,531 (100)
5×10^6 1-wk spleen cells	75 (3)	15 (2)	1,575 (10)	443 (10)
10^7 1-wk thymus cells	2,275 (83)	1,400 (194)	9,875 (63)	15,100 (300)

* Cells added with SRBC to cultures of 4×10^6 immune spleen cells.
‡ The "control" response is represented on line 1 of this table. The data in this table represent responses of pooled duplicate cultures in a single experiment. Similar relative results were obtained in a repeat of this experiment.

the nylon nonadherent spleen cells are always more suppressive than the nylon adherent cells. The 1-wk suppressor cell is also nonadherent to Sephadex G-10 or glass beads (Table VI).

The various populations of 1-wk spleen cells were then treated with anti-Thy.1.2 serum. As can be seen in Table VII, anti-Thy.1 treatment of unseparated 1-wk spleen cells actually enhanced the suppressive capacity of the treated cells. Similar results were obtained with anti-Thy.1.2 treatment of glass bead and nylon wool nonadherent 1-wk spleen cells. In these experiments, the same total numbers of treated and untreated cells were added as suppressors to the immune cell cultures. The increased suppression after this treatment suggests that anti-Thy.1 specifically removed a nonsuppressive cell. We have shown (14 and fn. 2) with adult suppressor cells that treated cells added to immune cell cultures in the same total numbers as untreated did not show significantly decreased suppressor activity but when added at the serum-depleted concentration, the suppression was at least partially abrogated. With adult suppressor

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TABLE V
Enrichment of 1-Wk Suppressor Cells by Passage Through Nylon Wool

Suppressor cell fraction*	Day 5 anti-SRBC PFC/culture (% control)‡			
	Experiment 1		Experiment 2	
	IgM	IgG	IgM	IgG
None	2,000 (100)	2,875 (100)	10,350 (100)	11,400 (100)
Unseparated	352 (18)	1,423 (49)	195 (2)	965 (8)
Nonadherent	163 (8)	145 (5)	90 (1)	657 (6)
Adherent	878 (44)	3,822 (133)	780 (8)	6,533 (57)

* 5×10^6 1-wk fractionated spleen cells added with SRBC to cultures of 4×10^6 immune adult spleen cells.
‡ The "control" response is represented on line 1 of this table.

TABLE VI
Adherence Properties of 1-Wk Suppressor Cells

Cells added*	Day 5 indirect PFC/culture (% control)‡	
	Experiment 1	Experiment 2
None	41,150 (100)	26,275 (100)
Whole	1,190 (3)	1,062 (4)
Nonadherent	683§ (2)	127‖ (1)

* 5×10^6 1-wk cells added with SRBC to cultures of 4×10^6 immune spleen cells.
‡ The "control" response is represented on line 1 of this table.
§ 1-wk cells nonadherent to Sephadex G-10.
‖ 1-wk cells nonadherent to glass beads.

cells, however, no enhancement of suppression was ever observed after treatment of the regulator cells with anti-Thy.1.2 (see footnote 2).

Fractionation of 1-wk Spleen Cells on Nylon Wool Columns. The 1-wk suppressor cell is enriched in the fraction of spleen cells that is nonadherent to nylon wool, yet also enriched in the anti-Thy.1 insensitive fraction. Control nylon wool separations of 1-wk spleen cells indicate that the resultant adherent and nonadherent fractions are not as clearly separated into B- and T-cell populations as in the adult (11, 16). Nylon nonadherent 1-wk cells are 50–60% Ig^+ by mixed antiglobulin rosette assays and are 20–50% anti-Thy.1.2 sensitive. They are still highly responsive to LPS (stimulation index of 21 as compared with an index of 14 for adherent cells), unlike adult nonadherent fractions which have LPS responses that are 11–17% of those of adherent cells. Furthermore, PHA and Con A responses are not enhanced as they are in the nonadherent fractions of adult spleen cells. Nylon adherent populations of 1-wk spleen cells contain 18–20% anti-Thy.1 sensitive cells and 79–86% Ig^+ cells, yet the mitogen responsiveness of this population is changed only slightly from the unseparated 1-wk cells and is not suggestive of a T- or a B-cell enrichment. There were also no clear morphological differences between the adherent and nonadherent cells observed in differential cell counts done on cytocentrifuge smears stained with Giemsa stain.

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TABLE VII
Enhanced Suppression of Antibody Responses by Normal Spleen Cells Treated with Anti-Thy.1.2 and Complement

Suppressor cell fraction added*	Day 5 anti-SRBC PFC/culture (% control)‡	
	IgM	IgG
Experiment 1		
None	600 (100)	41,150 (100)
Untreated	285 (48)	1,990 (3)
Anti-Thy.1.2 + C	15 (3)	383 (1)
Experiment 2		
None	768 (100)	25,732 (100)
NMS + C	168 (22)	2,007 (8)
Anti-Thy.1.2 + C	102 (13)	1,148 (4)
Experiment 3		
None	450 (100)	36,550 (100)
Untreated	143 (32)	3,026 (8)
Anti-Thy.1.2 + C	0 (0)	0 (0)

* 5×10^6 1-wk cells added with SRBC to cultures of 4×10^6 immune adult spleen cells.

‡ The "control" response is presented in the first line of each experiment where there were no suppressor cells added.

Discussion

We have demonstrated previously that adult cells in the presence of immune cells are active suppressors of both primary and secondary antibody responses (11). The present study indicates that 3-wk-old spleens have a lower potential for suppression than the adult spleens, suggesting the continuing maturation of this function after birth. The 1-wk-old spleens appear to have the same or greater potential for immune suppression as adult cells. The data suggest, however, that this cell acts differently and probably is a different cell from the adult suppressor cell. Like the adult specific suppressor cell, they are glass bead, Sephadex G-10, and nylon wool nonadherent and therefore probably are not macrophages and possibly are thymus-derived cells. Unlike the adult cells, they suppress nonspecifically, do not require stimulation from primed cells to suppress, are relatively anti-Thy.1.2 insensitive, and, in combination with immune cells, do not form the small indistinct plaques in assay as seen in other immune cell cultures.

The presence of tiny and indistinct plaques in assays of cultures suppressed with adult spleen cells and their absence from those suppressed with 1-wk cells suggests that these cells have different effects on the immune responses. This phenomenon may represent two different forms of suppression, suppression at the induction stage of the numbers of AFC generated by both adult and young suppressors and suppression at the effector stage of the amounts of antibody released by each AFC by the adult suppressors only. Evidence in another system for suppression of the amount of antibody produced has been described (24). More likely, the tiny plaques may represent anti-SRBC antibody cytophilically bound to Fc receptors on cells that are not synthesizing specific anti-SRBC antibody or antibody bound to SRBC or SRBC fragments from the

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culture challenge which may lyse the binding SRBC and some adjacent SRBCs in the assay layer. Young spleen cells may prevent, from the initiation of the cultures, the release of this antibody by the immune cells or its uptake by other cells. Adult cells are most suppressive in the later phases of the response (11). These differences then would support the notion that 1-wk suppressors are acting spontaneously, whereas adult cells act suppressively only after induction by an immune stimulus.

Mosier and Johnson (15) have previously described a nonspecific, nylon nonadherent, anti-Thy.1 sensitive cell present in the spleen and thymus tissues of newborn mice that is capable of suppressing primary antibody responses of adult spleen cells. The 1-wk suppressor cell described in the present paper is also nylon wool nonadherent, although this population is not as clearly enriched in T cells as the adult nonadherent cells. Other experiments argue against this cell being thymus derived. Standard treatment with anti-Thy.1 was ineffective in removing the suppression and in fact resulted in the enhancement of suppression. The cell suppressive of the primed cell response also was absent from the 1-wk thymus. Recently, Mosier et al. (25) have shown that a thymocyte population representing about 5% of the total, which is resistant to mouse thymic virus, is enhanced in suppressor activity. These cells exhibit decreased sensitivities to anti-Thy.1 and anti-Ly antisera relative to the rest of the thymocytes. The enhanced suppressor activity in 1-wk cells in the present experiments may result from similar cells which selectively escape the antiserum treatment.

The unfractionated thymocytes may not be able to suppress the secondary response used in this study as easily as the primary response, particularly in view of the very low concentration of suppressor cells in the population (25).

Neonatal or fetal lymphocytes also effectively inhibit the in vitro division of adult lymphocytes both in unstimulated cultures or in the presence of PHA (26). Although newborn cells are extremely suppressive in vitro, in vivo suppression in these animals has not been demonstrated, possibly because of differences in cell ratios or geography. Adult cells injected into newborns do not seem to be inhibited in their immunologic activities. This has been demonstrated with graft vs. host disease when allogeneic adult cells are injected into newborn animals (27). Also, newborns do not suppress the secondary antibody responses of adult immune syngeneic cells adoptively transferred with antigen into the young mice (28). This is in contrast to adults in which adoptively transferred immune cells do not reach the levels of secondary antibody production that they do in the original donor, or in newborn hosts.

The data presented here suggest that the numbers of nonspecific suppressor cells diminish with age. However, they may be present in low numbers in the adult and may be the cells stimulated with Con A (6) or allogeneic cells (29). The nonspecific suppressor is possibly being replaced by specific suppressor cells, which reach adult levels sometime after 3 wk of age. Although these data do not prove the absence of specific suppression in newborn animals, they do indicate a preponderance of nonspecific suppression, making specific suppressor cell activity undetectable. The demonstration that the level of suppression in 3-wk-old animals is lower than that in adults is suggestive of a maturation of the

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specific suppressor cells and a disappearance of the nonspecific suppressor cells. In ontogeny, nonspecific suppressor cells may play a role in the natural induction of tolerance to self or the ease of experimental induction of tolerance (30) in newborn animals. The preponderance of suppression in the newborn may be the result of an enhanced number of suppressor cells or an absence of helper cells and therefore a relatively larger number of suppressor cells (31). Teleologically, it would seem logical that the development of specific suppressor cells as seen in adults would be necessary only with the development of the ability to mount specific immune responses, possibly as a means to turn off those same responses.

Summary

The activity of suppressor cells from spleens of mice of varying ages was assessed by their addition to cultures of normal or SRBC immune spleen cells together with a challenge of SRBC. 1-wk and adult spleen cells were highly suppressive of the secondary in vitro antibody response to SRBC. 3-wk spleen cells were less active in suppressing this response. The nature of the suppression and the character of the suppressor cells changed in this period. Whereas adult spleen cells demonstrated specificity, 1-wk cells nonspecifically suppressed all responses tested. Further, unlike adult suppressor cells (which are Thy.1.2 positive), 1-wk suppressor cells are insensitive to anti-Thy.1.2 treatment in this system. Both cells are nonadherent to glass beads and nylon wool and are undetectable in the normal thymus.

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