Ly PHENOTYPE AND MECHANISM OF ACTION OF MOUSE NEONATAL SUPPRESSOR T CELLS

By D. E. MOSIER, B. J. MATHIESON,* AND P. S. CAMPBELL

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

Antibody production during the neonatal period appears to be under more stringent regulation than during adult life in the mouse. Cultures of neonatal mouse spleen cells are prevented from fully expressing their B-cell functional potential by the activity of suppressor cells. These cells were tentatively identified as T cells on the basis of their sensitivity to high concentrations of anti-Thy 1.2 (anti-θ) serum and complement or their failure to adhere to nylon wool columns (1). T lymphocytes can now be further divided into functional subpopulations on the basis of their surface Ly antigen expression. It has been reported, for example, that helper T cells are Ly 1^+, Ly $2,3^-$ while cytotoxic killer T cells are Ly 1^-, Ly $2,3^+$ (2–6). Suppressor T cells found in the adult mouse spleen appear to express only the Ly 2,3 phenotype (7–13). We have therefore attempted to isolate enriched populations of neonatal suppressor cells from mouse spleen and thymus and characterize these cells both for functional activity and surface expression of Ly 1, Ly 2, TL, Thy 1, and H-2 antigens. Cells were also characterized as to size and DNA content by acridine orange staining and automated cytofluorimetry (14). Enrichment of suppressor T cells was achieved by infection of newborn mice (less than 18 h old) with the herpes virus of mice, mouse thymic virus (15–18). The residual T cells, obtained 10–14 days after infection, were enriched for suppressor activity while they were depleted for most other T-cell functions such as helper activity, mixed lymphocyte and cytotoxic T-cell reactivity, and T-cell mitogen responses (16). This mouse thymic virus-resistant T-cell population, which represents about 5% of the normal T-cell number, thus served as a relatively homogenous preparation high in suppressor activity. It was this population which was analyzed in detail for surface phenotype and mechanism of action.

Materials and Methods

Mice. All mice used in these experiments were (C57BL/6N × DBA/2N) F_1 (BDF$_1$)[1] obtained from the Small Animal Section, Division of Research Services, National Institutes of Health. Pregnant C57BL/6N females were delivered to our animal facility and the F_1 offspring reared in laminar flow cabinets in semi-isolation. Mice of the X-linked mutant CBA/N strain crossed with

---

DBA/2N males were used to breed the (CBA/N × DBA/2)F$_1$ male (defective) and F$_1$ female (normal) mice used in some experiments.

Culture Conditions. Single cell suspensions of mouse thymus or spleen were prepared and cultured by using a microculture adaptation of the Mishell and Dutton method as previously described (19). Briefly, a total of 1 × 10^6 cells were cultured in each well of a flat-bottomed microtiter plate (Falcon 3040, Falcon Plastics, Division of BioQuest, Oxnard, Calif.) for periods of 3–4 days in a humidified 5% CO$_2$–95% air atmosphere. Antibody formation against the trinitrophenyl (TNP) determinant was stimulated by the addition of TNP$_{58}$ aminoethylcarbamylmethyl (aecm)$_{83}$ Ficoll, TNP-lipopolysaccharide (TNP-LPS), or TNP-*Brucella abortus*. These antigens appear to be thymic-independent in the newborn as well as the adult mouse (D. E. Mosier. Manuscript in preparation) and their preparation has been described previously (20, 21). Direct plaque-forming cells (PFC) were assayed against TNP-modified sheep erythrocytes prepared as described by Rittenberg and Pratt (22). All groups were assayed in triplicate and the data logarithmically transformed to obtain the geometric mean. Data are expressed as the antilog of the geometric mean ×/÷ the relative standard error (the antilog of the log standard error). Proliferation assays were performed as described previously.

Virus Injection. Mouse thymic virus was obtained by serial passage from a stock maintained by Dr. Wallace Rowe, National Institute of Allergy and Infectious Diseases, National Institutes of Health and donated to us by Dr. Sue Cross. Newborn mice were injected intraperitoneally within 18 h of birth with a 1:10 dilution of a clarified supernate of virus thymus homogenate. Mice were examined when sacrificed 10–14 days later for the gross thymic necrosis associated with virus infection, and any mice not showing this sign were excluded from the experiment.

Cytotoxicity Assay. The complement-dependent cytotoxicity assay of Gorer and O'Gorman (23) was used with the modification described by Boyse et al. (24). Equal 0.05-ml columns of: (*a*) antiserum serially diluted; (*b*) selected rabbit serum diluted 1:15 in anti-Ly tests (25, 26), or guinea pig serum diluted 1:4 in anti-TL, -Thy 1, and H-2 tests (both complement sources were selected for low cytotoxicity against mouse thymocytes and high complement activity); and (*c*) cells suspended in medium 199 (Hank's balanced salt solution base) at 5 × 10^6/ml were incubated at 37°C for 45 min. Cell viability was assessed by trypan blue exclusion.

Antisera and Immunofluorescence Reagents. The anti-Ly, anti-TL, and anti-Thy 1.2 sera used in this study were obtained from Doctors E. A. Boyse and F.-W. Shen and details of their specificity are presented elsewhere.[2] The anti-H-2^b reagent was obtained from BALB/c AnN female mice which had received both skin grafts and peripheral lymphoid cell suspensions from (BALB/c × C57BL/6N)F$_1$ or C57BL/6 female mice. At the dilutions used in these studies, the antiserum does not react with BALB/c, NZB, or B10.D2 mouse thymocytes. The anti-Thy 1.2 serum used for bulk killing of neonatal spleen cell suspensions was a conventional AKR/J anti-C3H/J thymocyte reagent obtained in the ascites form from Dr. I. Zitron and used after absorption with AKR thymocytes and ultracentrifugation at 100,000 g for 20 min.

The immunofluorescence assays employed a polyvalent goat antiserum to mouse immunoglobulin. Antibody from this antiserum was purified by affinity chromatography on a column containing equal amounts of γ$_1$, γ$_{2a}$, and γ$_{2b}$ mouse myeloma proteins. The purified antibody reacted in double diffusion gels with all classes of immunoglobulins and with κ but not λ chains. Goat antibodies to mouse immunoglobulins (anti-mIg) were coupled to fluorescein by reaction with fluorescein isothiocyanate to achieve a molar ratio of fluorescein to protein of 2.5–5.7. Goat anti-mIg were conjugated with rhodamine by reaction with tetramethylrhodamine isothiocyanate isomer R (lot F6 EHRT 12, BBL) to achieve a rhodamine:protein molar ratio of 3.0. Unconjugated fluorescein or rhodamine were removed by passage over Sephadex G-25 equilibrated with 0.15 *M* NaCl, pH 7.4. (Pharmacia Fine Chemicals, Piscataway, N. J.) Rhodamine-anti-mIg also was passed over activated charcoal. These two fluorescent reagents are designated fl-anti-mIg and rh-anti-mIg, respectively.

Immunofluorescence Assay. The immunofluorescence test used with the antisera to Ly determinants and the details of the antisera specificity are reported elsewhere.[2] A cell suspension containing 1 × 10^6 cells was prepared in Hanks' MEM containing 10% heat-inactivated fetal calf serum and 0.1% sodium azide, and incubated with the appropriate antisera for 30 min on ice. The

---

[2] Mathieson, B. J., P. S. Campbell, M. Potter, and R. A. Asofsky. 1977. Expression of differentiation antigens on mouse lymphomas. Manuscript submitted for publication.

TABLE I

Mitogen Responses of Normal or Mouse Thymic Virus-Infected 11-Day Old BDF₁ Thymus and Spleen Cells

| | Mitogen: | Geometric mean cpm, ×/÷ relative SE | | | |
		0	PHA	Con A*	LPS
Cells:					
Normal thymus		182	1,604	33,074	1,586
		(1.25)	(1.11)	(1.08)	(1.11)
Virus-infected thymus		314	7,041	59,520	828
		(1.02)	(1.11)	(1.20)	(1.17)
Normal spleen		904	6,422	29,547	5,126
		(1.28)	(1.18)	(1.17)	(1.02)
Virus-infected spleen		1,169	894	4,406	37,533
		(1.15)	(1.12)	(1.08)	(1.05)

* Con A, concanavalin A.

cells were washed twice, and the pellet resuspended in the first fluorescent reagent. The sequence was reported for double staining experiments.

Cytofluorimetry. Rapid flow cytofluorimetry was performed after staining of cell suspensions with 1 μg/ml acridine orange (14) by using a model 4800 A Cytofluorograf (Bio Physics Systems, Inc., Mahopac, N. Y.) and data collected by using a 100-channel distribution analyzer. Size estimations are based on light scattering measurements performed with the Cytofluorograf.

Mitogen Responses. Cells were stimulated with phytohemagglutinin-P (Burroughs Wellcome & Co., Tuckahoe, N. Y.), concanavalin A (Sephadex-purified, Pharmacia Fine Chemicals), or lipopolysaccharide (from *Escherichia coli* 0111:B4, Westphal procedure of extraction, Difco Laboratories, Detroit, Mich.), cultured for 68–70 h, pulsed with 1 μCi [³H]thymidine (6.0 Ci/mM, New England Nuclear, Boston, Mass.) for 4 h, and samples collected with an automated cell harvester (MASH II, Microbiological Associates, Bethesda, Md.).

Results

Neonatal Infection with Mouse Thymic Virus Spares Suppressor Cell Activity in Thymus and Spleen. Neonatal infection of BALB/c mice with mouse thymic virus previously has been shown to decrease most, but not all, T-cell functions within 1 wk of infection (16). Among the functions resistant to the effects of viral infection are phytomitogen responsiveness in the thymus (but not the spleen) (16), the induction of T-cell-mediated suppression of pneumococcal polysaccharide responses (18), and the ability to synergize with effector cells in the graft-versus-host reaction (17). Newborn BDF₁ mice were injected within 18 h of birth with mouse thymic virus. 10–16 days later, residual cells from their thymuses and spleens were recovered. Thymuses from virus-infected mice yielded 5–10% of the number of cells recovered from uninfected age-matched controls while spleen cell recovery was about 50% of normal. To confirm the differential effect of mouse thymic virus on thymus and spleen T cells, recovered cells were stimulated with Phytohemagglutinin (PHA), Con A, or LPS and [³H]thymidine incorporation determined after 72 h of culture. As shown in Table I, the virus infection had moderately increased the response of residual thymocytes to PHA and Con A, but residual spleen cells had much reduced or absent proliferative responses to both mitogens. The response of splenic B cells to LPS was increased in the cells from viral-infected mice, but the magnitude of the increase was not usually as great as in this particular experiment. Cells from the same group of animals were mixed with graded numbers of adult BDF₁ spleen cells and the
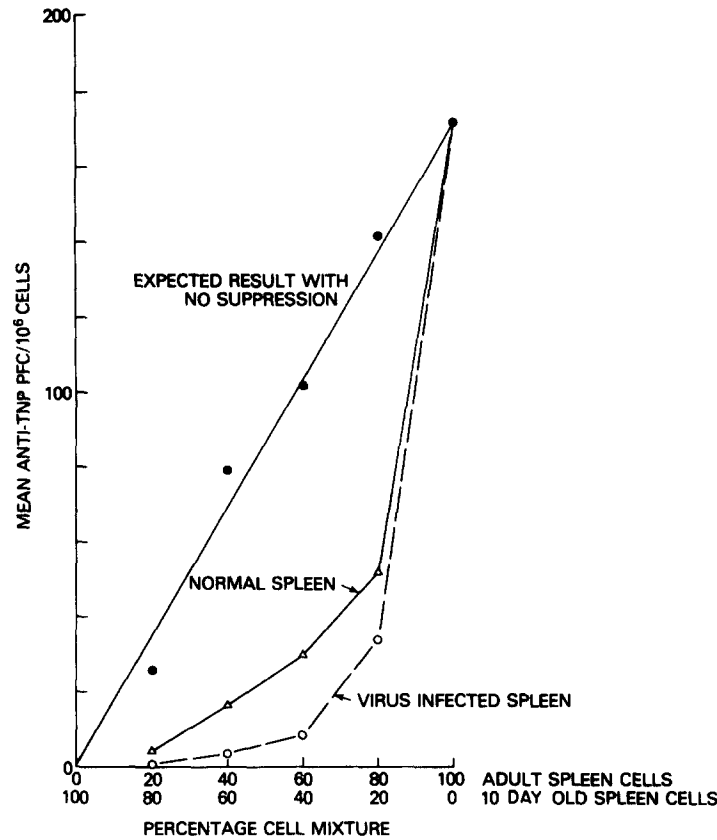
FIG. 1. The in vitro anti-TNP PFC response of adult BDF_1 spleen cells stimulated with TNP-Ficoll in the presence of normal or mouse thymic virus-infected 10-day old BDF_1 spleen cells. The solid straight line is the regression line of the PFC response of adult spleen cells cultured alone. In all other instances, the total number of cells per microculture was 1×10^6. The suppression of the PFC response was statistically significant ($P < 0.05$) at all concentrations of neonatal cells.

mixture of cells stimulated with TNP-Ficoll, a thymus-independent antigen. Over the range of cell numbers employed, the number of adult spleen cells cultured was directly and linearly related ($r = 0.97$ in three replicate experiments) to the numbers of PFC's generated after 4 days of culture. Fig. 1 shows the results of adding spleen cells from 10-day old normal or virus-infected BDF_1 mice to adult BDF_1 spleen cells with the total cell number per microculture held constant at 1×10^6. The ability of spleen cells from the virus-infected mice to interfere with the B-cell triggering induced by TNP-Ficoll was even greater than that of normal 10-day old spleen cells. That this suppression was a result of residual T cells and not a direct effect of mouse thymic virus was shown by demonstrating that: (a) the suppression was still sensitive to treatment of spleen cells with undiluted anti-θ serum and complement, and (b) direct addition of mouse thymic virus preparation or supernates of spleen cells from infected mice were without suppressive effects (data not shown). The thymocytes which survive mouse thymic virus infection also were shown to be more suppressive of B-cell activation than age-matched control thymocytes (Table II).

TABLE II

Modification of Adult Spleen Anti-TNP PFC Responses By Neonatal Thymocytes

Modifier cells: (1 × 10⁵) Antigen:	Geometric mean PFC/10⁶ cells, ×/÷ relative SE*		
	None	Normal thymus	Virus-infected thymus
0	0	0	0
TNP-*Brucella abortus*	284	90	50
	(1.03)	(1.02)	(1.08)
TNP-LPS	181	117	64
	(1.15)	(1.08)	(1.28)
TNP-Ficoll	58	17	7
	(1.04)	(1.41)	(1.87)

* Responding cells were 1 × 10⁶ adult BDF₁ spleen cells per microculture. Thymocytes were from normal or neonatally mouse thymic virus-infected 10-day BDF₁ mice. TNP-*Brucella abortus* and TNP-LPS responses were assayed after 3 days of culture and TNP-Ficoll responses after 4 days of culture by determination of direct hemolytic PFC versus TNP-sheep erythrocytes.

This subpopulation of virus-resistant T cells therefore seems to be increased in suppressor activity while previous studies have shown it to be devoid of most other T-cell functions. The thymus and spleen cells of neonatally mouse thymic virus-infected BDF₁ mice were chosen on this basis as a relatively homogenous population for analysis of cell surface phenotype and morphology.

Ly 1, Ly 2, Thy 1, TL, and H-2 Phenotype of Mouse Thymic Virus-Resistant Thymocytes. The population of cells obtained from the thymuses of BDF₁ mice injected as neonates with mouse thymic virus (and demonstrated in parallel experiments to be enriched for suppressor activity) were tested both by cytotoxicity and immunofluorescence assays for expression of mouse cell surface alloantigens. In each experiment, cytotoxicity assays or immunofluorescence labeling was performed in parallel on age-matched normal thymocytes as well as mouse thymic virus-resistant thymocytes. Susceptibility of the two cell populations to killing or staining by anti-Ly antisera first was assessed. Since C57BL/6 and DBA/2 mice express different Ly alleles, BDF₁ cells were susceptible to antiserum of both allelic specificities. Treatment of normal 12-day old BDF₁ thymocytes with anti-Ly 1.1 or anti-Ly 1.2 antiserum and complement resulted in 60–70% cytotoxicity, as did exposure to anti-Ly 2.1 or anti-Ly 2.2. Fig. 2 shows representative cytotoxicity data from one of four replicate experiments by using the anti-Ly 1.1 or anti-Ly 2.1 reagents. By contrast, between 20 and 40% of thymocytes from virus-infected mice were susceptible to lysis either by anti-Ly 1.1 (or 1.2) or by anti-Ly 2.1 (or 2.2) antiserum (Fig. 2). The reduced sensitivity of virus-resistant thymocytes to anti-Ly 1 or anti-Ly 2 killing suggests that this subpopulation has either fewer cells expressing Ly antigens or that the amount of Ly antigen expression per cell is substantially reduced. When normal or virus-resistant cells were exposed simultaneously to both anti-Ly 1.1 and anti-Ly 1.2 antiserum, more than 80% of both cell populations were susceptible to lysis (Fig. 4). The same finding was obtained with simultaneous killing with anti-Ly 2.1 and 2.2 (data not shown). These data lead to the tentative conclusion that normal and virus-resistant thymocytes both are Ly 1- and Ly 2-positive although the absolute amount of Ly antigen expressed per cell seems to be
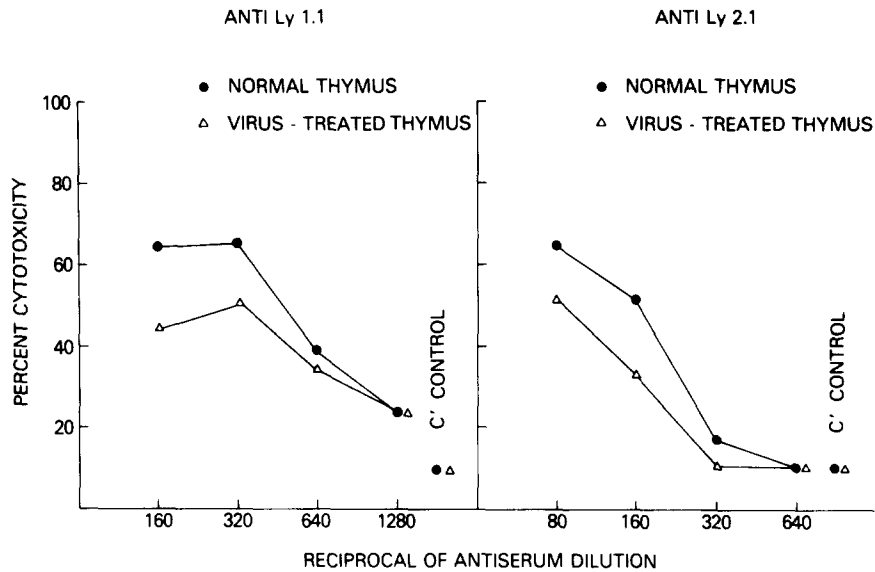
Fig. 2. Cytotoxicity with anti-Ly 1.1 and anti-Ly 2.1 sera. Thymus cell suspensions pooled from three to four thymuses of virus-treated and normal BDF_1 mice were incubated with antiserum and C and cytotoxicity (percent dead cells, stained with trypan blue) was determined. Anti-Ly 1.1 serum (B6 × BALB/c)F_1 anti-B6/$Ly 1.1$ normal thymus, or anti-Ly 2.1 serum, B6/H-2^k anti-CE normal thymus, was used with selected rabbit serum ($^1/_{15}$) as a source of C. Antisera were diluted to a point where maximal kill was still observed on normal F_1 thymus cell suspensions.

reduced in the virus-resistant subpopulation. This conclusion was confirmed directly by two indirect immunofluorescence studies, one of which is shown in Table III. Normal thymocytes or virus-resistant thymocytes were exposed to anti-Ly antisera, washed, and subsequently treated with a fluorescein- or rhodamine-conjugated anti-mIg reagent. Such treatment demonstrated that more than 90% of both cell populations could be stained with anti-Ly 1, 2, or 3. Cells were stained with anti-Ly 1 and fluorescein-anti-mIg followed by anti-Ly 2 (or 3) and rhodamine-anti-mIg to determine if both Ly determinants were present on a single cell (as would be inferred from the high percentage of cells positive with either reagent). Such double staining experiments were performed (Table III) and directly confirmed the presence of Ly 1, 2, and 3 determinants (with a somewhat less intense staining then controls) on virus-resistant thymocytes. The Ly phenotype of that population and the suppressor cells contained within it is therefore Ly 1^+, Ly $2,3^+$.

Treatment of the same two cell populations with anti-TL, anti-Thy 1.2, and anti-H-2^b antisera and assessment of quantitative cytotoxicity (Figs. 3 and 4) led to the conclusion that the mouse thymic virus-resistant cells were less susceptible to killing with anti-TL and anti-Thy 1.2 sera and more susceptible to killing with anti-H-2^b serum than were normal thymocytes. We would infer that the altered susceptibility to these antisera is correlated with an altered expression of the antigens at the single cell level, but such data are consistent also with population shifts in antigen expression. These experiments, taken together, suggest that the suppressor function which we have studied is a property of cells

TABLE III

Mouse Thymic Virus-Resistant Thymocytes are Ly 1,2,3$^+$ By Double Immunofluorescence Staining

First reagent(s)	Second reagent(s)	Percent positively stained cells and color	
		Normal thymocytes	Virus-resistant thymocytes
Single staining			
anti-Ly 1.2	fl-anti-mIg*	91% ++‡ (green)	90% + (green)
anti-Ly 1.1	fl-anti-mIg	95% +++ (green)	95% ++ (green)
anti-Ly 2.2	rh-anti-mIg§	90% +++ (red)	90% + (red)
anti-Ly 2.1	rh-anti-mIg	90% ++ (red)	90% + (red)
anti-Ly 3.2	fl-anti-mIg	>95% +++ (green)	>95% ++ (green)
Double straining controls			
anti-Ly 1.2 +			
fl-anti-mIg	rh-anti-mIg	90% ++ (green only)	90% + (green only)
anti-Ly 1.1 +			
fl-anti-mIg	rh-anti-mIg	>95% ++ (green only)	90% + (green only)
anti-Ly 3.2 +			
fl-anti-mIg	rh-anti-mIg	>95% +++ (green only)	>95% ++ (green only)
Double staining			
anti-Ly 1.2 +	anti-Ly 2.2 +		
fl-anti-mIg	rh-anti-mIg	>95% +++ (red-green)	>95% ++ (red-green)
anti-Ly 3.2+	anti-Ly 2.2 +		
fl-anti-mIg	rh-anti-mIg	>95% +++ (red-green)	>95% ++ (red-green)
anti Ly 1.1 +	anti-Ly 2.1 +		
fl-anti-mIg	rh-anti-mIg	>95% +++ (red-green)	>95% ++ (red-green)

* Fluorescein-conjugated goat anti-mouse immunoglobulin; <1% of normal or virus-infected thymocytes stain with this reagent, or with its rhodamine-conjugated counterpart, or after exposure to both reagents.

‡ Number of ++'s indicates staining intensity.

§ Rhodamine-conjugated goat anti-mouse immunoglobulin.

within a subpopulation of cortical thymocytes with the following surface antigen phenotype: Ly 1$^+$, Ly 2, 3$^+$, TL$^+$, Thy 1$^+$ ↓, H-2$^+$ ↑.

Size and Distribution of Mouse Thymic Virus-Resistant Suppressor T Cells. Thymocytes from normal or virus-injected 10- to 13-day old BDF$_1$ mice were analyzed for size and DNA content by using rapid-flow cytofluorimetry. Individual thymic lobes also were prepared for light and electron microscopy. The 5% of thymocytes surviving neonatal infection with mouse thymic virus were characterized by a larger size, greater DNA content by acridine orange staining (Fig. 5), and a localization to the subcapsular outer cortex of the thymus. This subpopulation represents a minor fraction of cells present in the normal thymus and thus seems to have been selected for rather than induced by the cytolytic virus infection. From its size and high DNA content the virus-resistent subpopulation is rapidly dividing with many cells in late S or G$_2$ phases of the cell cycle.

The Mechanism of Action of Neonatal Suppressor T Cells. The final set of experiments to be presented here concern the mechanism of the suppression of antibody formation induced by the cells characterized above. These experiments were designed to decide between two alternative modes of suppression; the first would be a reduction in the number of B cells initially triggered by a T-independent antigen (TNP-Ficoll), and the second would be a regulation of B-
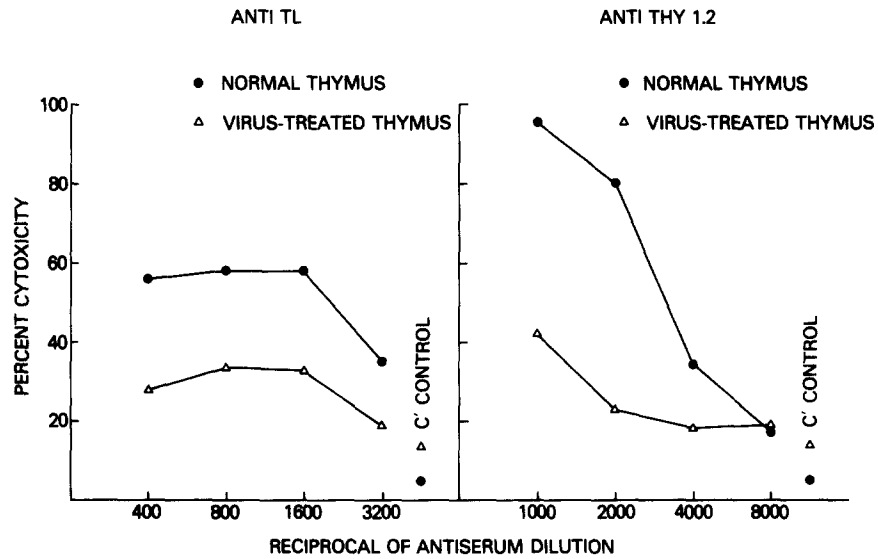
ANTI TL   ANTI THY 1.2

Fig. 3. Cytotoxicity with anti-TL and anti-Thy 1.2 sera. Thymus cell suspensions from pooled thymuses of virus-treated or normal BDF_1 mice were incubated with antiserum and C. Anti-TL serum, (B6 × A/TL$^-$)F_1 anti-ASLI (an A strain tumor), or anti-Thy 1.2 serum (A/Thy 1.1 × AKR/H-2^b)F_1 anti-ASLI, was used with selected guinea pig serum ($^1/_4$) as a source of C. Cytotoxicity with anti-TL on normal DBA/2 thymocytes (TL.2$^+$) is 70% at 1/400.

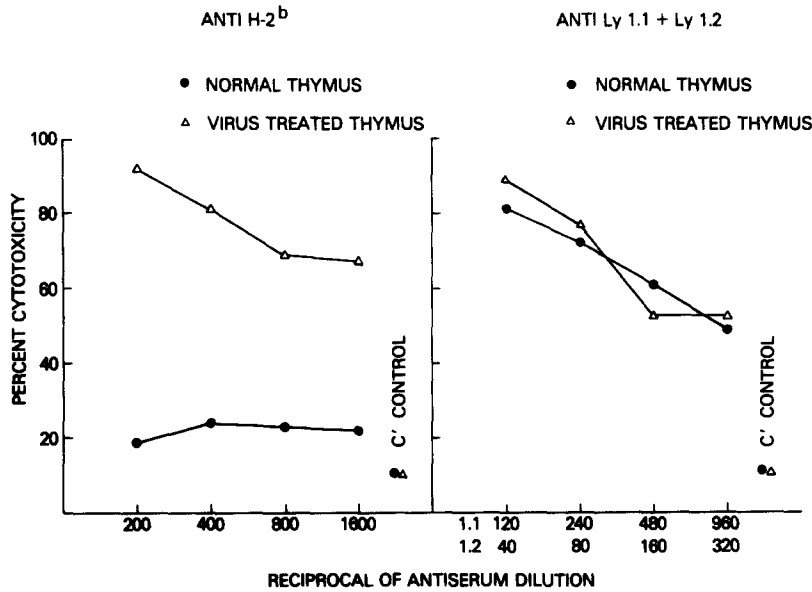ANTI H-2^b ANTI Ly 1.1 + Ly 1.2

Fig. 4. Cytotoxicity with anti-H-2^b and anti-Ly 1.1 + anti-Ly 1.2 sera. Thymus cell suspensions from pooled thymuses were treated with C and serum as indicated in Fig. 2 for anti-Ly sera and as indicated in Fig. 3 for anti-H-2. BALB/c AnN mice were immunized with skin grafts and lymphoid cell suspensions from B6 or (B6 × BALB)F_1 mice to produce a multispecific anti-H-2^b. Anti-Ly 1.1 was mixed with anti-Ly 1.2, C3H/An anti-CE normal thymus, to yield the dilutions indicated on the abscissa. A single incubation with mixed antiserum and C was then performed.
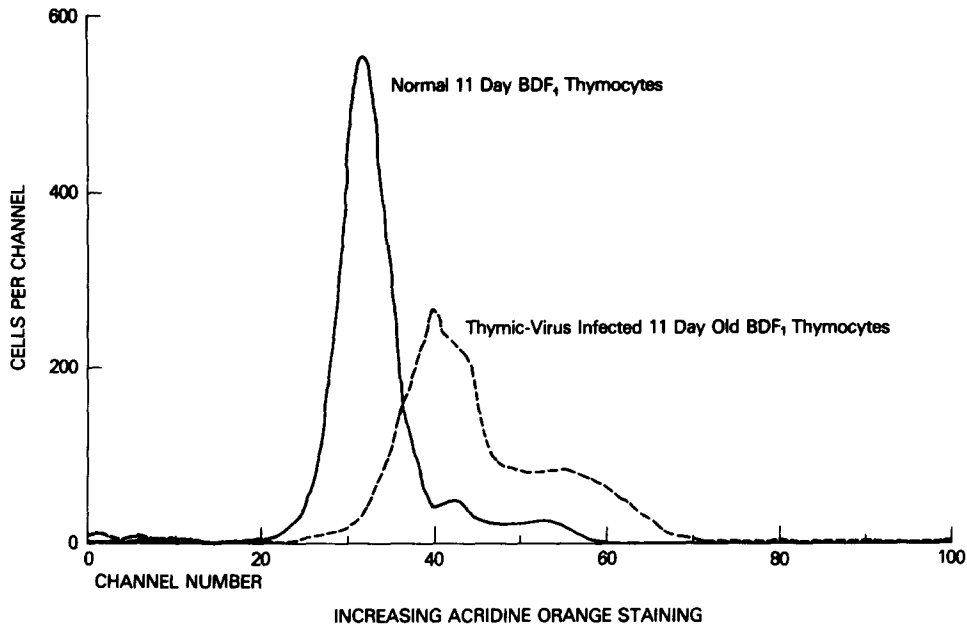
FIG. 5. The green fluorescence staining pattern of 11-day old normal or mouse thymic virus-infected BDF_1 thymocytes stained with acridine orange. A total of 1×10^4 cells were analyzed on the Cytofluorograf and the number of cells in each of 100 channels (fluorescence intensity increasing from 1 to 100) is plotted. Under these conditions, staining intensity primarily is determined by DNA content per cell.

cell differentiation or proliferation. The suppressor cells induced by concanavalin A treatment of adult spleen cells seem to act by the latter mechanism (27) and limit the burst size of each B-cell clone. An experiment designed to determine the number of adult splenic B cells triggered under limiting conditions by TNP-Ficoll in the presence or absence of neonatal suppressor cells is depicted in Fig. 6. This experiment is predicated on the finding that $(CBA/N \times DBA/2)F_1$ male spleen cells have an X-linked defect that is limited to their B cells and totally precludes any TNP-Ficoll response (28–30). Such defective F_1 male mice have no known T-cell abnormalities including no known difference in suppressor cell function (30). Syngeneic $(CBA/N \times DBA/2)F_1$ female spleen cells have normal in vivo and in vitro PFC responses to TNP-Ficoll. Responsive adult F_1 female cells were serially diluted into unresponsive F_1 male adult spleen cells (lacking neonatal suppressors) or 10-day old neonatal F_1 male spleen cells (possessing neonatal suppressor cells) and the mixture stimulated with TNP-Ficoll. The number of cells per culture was constant at 1×10^6. The TNP-specific PFC response per F_1 female B cell added to each culture was reduced significantly by dilution into neonatal rather than adult F_1 male cells. This result suggests that the suppressive effect of the neonatal spleen cells was due to a reduction of functional B-cell precursor frequency. The alternative possibility, that neonatal cells suppress burst size in a linear dose-dependent fashion, was excluded by the following experiment. Adult BDF_1 spleen cells were cultured in the presence or absence of 1×10^5 (10%) mouse thymic virus-resistant spleen cells and the TNP-Ficoll PFC response determined after 40, 48, and 62 h of
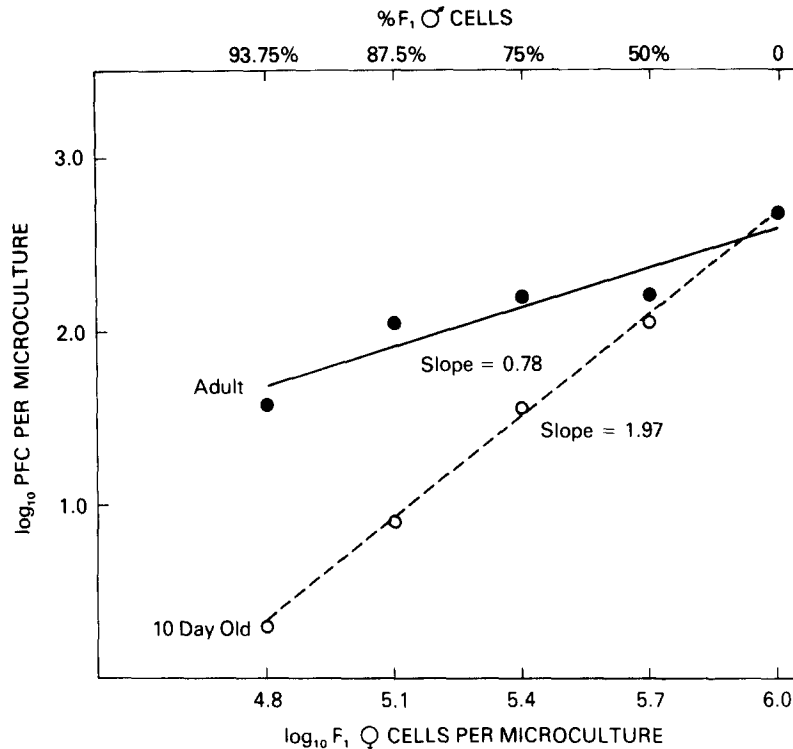
%F, ♂ CELLS

FIG. 6. The anti-TNP PFC response ($\log_{10}$ converted) of adult normal (CBA/N × DBA/2)F$_1$ female spleen cells serially diluted into genetically unresponsive (CBA/N × DBA/2)F$_1$ male adult (solid line) or 10-day old (broken line) spleen cells. The antigen was TNP-Ficoll, to which F$_1$ male cells are unresponsive because of an X-linked defect in B-cell development. No T-cell abnormalities are known in such mice. Such genetically unresponsive cells therefore are ideal sources of neutral filler cells for cell dilution experiments, as demonstrated by the slope (0.78) of the response per input cell approaching the theoretical limit of one for a single hit phenomenon. Dilution of adult cells into unresponsive neonatal syngeneic cells resulted in a decrease in the number of precursor cells triggered that was proportional to the number of neonatal cells.

culture, before the peak response at 96 h. As shown in Fig. 7, the anti-TNP PFC response is equally suppressed at all time points early in the response. This result is in direct contradistinction to the expected effect of reduction in burst size which would be to show increasing suppression as the duration (and magnitude) of the in vitro response increases. Taken together these two experiments indicate that the main effect of neonatal suppressor T cells is to reduce the number of B cells initially triggered by antigen, and that once triggering has occurred no further suppressive effects are manifested.

Discussion

In these studies we have characterized a subpopulation of lymphoid cells in the neonatal thymus which have been shown to possess marked suppressor activity for antibody synthesis. Such suppressor function is also present in the neonatal spleen and presumably reflects the activity of the same peripheralized
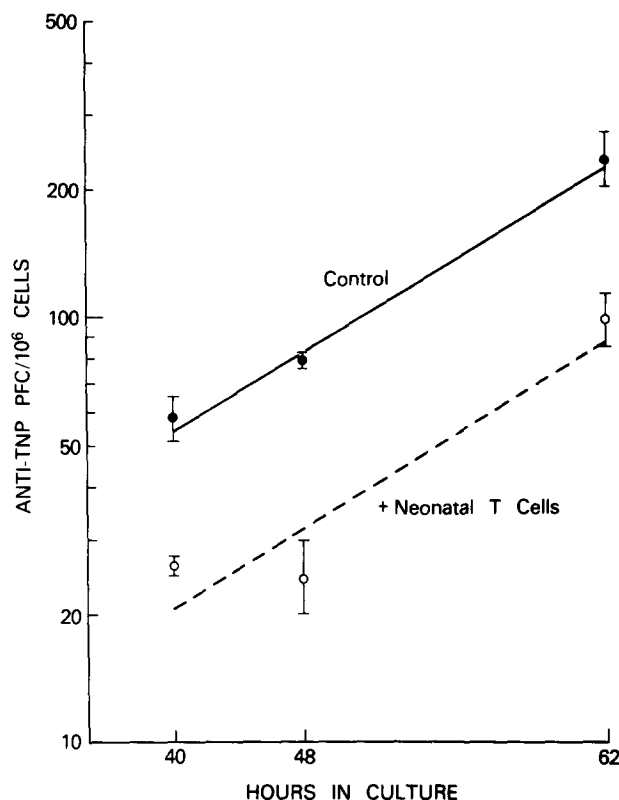
Fig. 7. The anti-TNP PFC response of adult BDF_1 spleen cells cultured in the presence or absence of 12-day old mouse thymic virus-resistant syngeneic spleen cells. The antigen used was TNP-Ficoll and direct PFC were measured at 40, 48, and 62 h of culture. The regression coefficient for the control response is 0.98 and for the suppressed response is 0.87. The slopes of the two lines are identical.

subpopulation of cells, but splenic neonatal suppressor T cells were not analyzed in detail for their surface phenotype because they represent such a small fraction of cells in the spleen (even in neonatally mouse thymic virus-infected mice). The method used to yield a relatively pure, homogenous subpopulation of thymocytes was neonatal infection with mouse thymic virus, an agent previously shown to produce a cytolytic infection of the thymus 7-10 days after injection (15). The 5% of outer cortical thymocytes resistant to the virus were demonstrated to: (a) have greater suppressor activity than cells from intact thymuses, (b) have a Ly 1^+, $2,3^+$, TL^+, Thy 1^+ ↓, $H-2^+$ ↑ surface alloantigen phenotype; and (c) to be large cells with a greater than average DNA content. From this data we would suggest that the suppressor activity found in neonatal T cells (both in the thymus and probably in the spleen) is a property of large, rapidly dividing cells localized in the outer cortex of the thymus and, by inference, a similar population of cells in the spleen. This inference is based on the absence of T lymphocytes with high Thy 1 expression in the T-dependent areas of splenic lymphoid follicles after neonatal mouse thymic virus infection despite an increase in anti-Thy 1 sensitive suppressor activity. Although we

were unable to directly test the Ly phenotype of the splenic suppressor T cell, we also would infer that it is Ly 1^+, Ly $2,3^+$ as is the suppressor cell found in the thymus.

A population of thymocytes with similar phenotype, size, and localization has been described (31). On the basis of early incorporation of [^{3}H]thymidine after topical labeling, this cell is assumed to be rapidly dividing and present in the subcapsular region. It has been suggested that such cells are precursors for more mature T lymphocytes, but it would seem inappropriate to call a cell expressing at least two differentiated functions, suppression, and augmentation of a graft-versus-host reaction, immature. If identical cells are found in the spleen, as the presence of suppressor activity would suggest, then such cells must have emigrated from the thymic cortex or have undergone partial differentiation in a nonthymic environment (32). The finding of T cells capable of suppressing B-cell activation in the spleens of nude mice (33) provides some support for the latter concept.

The suppressor T cells found in the thymus and spleen of neonatal mice seem to differ from T cells capable of suppressing immune response in adult animals. Most authors (7–13) have found antigen- or mitogen-induced suppressor cells to express the Ly 1^-, Ly $2,3^+$ phenotype rather than to be Ly 1^+, Ly $2,3^+$. Secondly, we have no indication that the administration of antigen or stimulation with concanavalin A (under circumstances which induce suppression in adult spleen cells) has any effect on neonatal suppressor cells (D. E. Mosier and E. Goldings. Unpublished observations). Whether neonatal Ly $1,2,3^+$ cells give rise to adult Ly $2,3^+$ suppressors is unclear.

We have argued previously that neonatal suppressor T cells must directly suppress adult B cells since thymus-independent responses are subject to suppression. This interpretation is strengthened by the experiments depicted in Figs. 6 and 7, which strongly suggest that neonatal suppressor T cells directly react with precursor B cells and prevent antigen-induced differentiation into antibody-forming cells. The data do not exclude, however, an indirect mechanism of suppression mediated, for example, via macrophages or another T-cell subpopulation which has the same phenotype as the T cells which initiate suppression. If B cells are directly subject to suppression, then neonatal suppressor T cells stand in contrast to adult suppressor systems (11) where T cells seems to be the targets of suppressive phenomena. The characterization of the suppressive subpopulation in the thymus suggests one intriguing mechanism for suppression for which we can adduce some very indirect evidence. It has been reported that neonatal thymocytes react by proliferation when confronted with irradiated or mitomycin-treated syngeneic adult spleen cells, generating what has been termed an isogeneic lymphocyte reaction (34). Furthermore, it was shown that the target cells were mature B cells (35). In our experience, this phenomenon is limited to a few strains (notably, CBA) and is quite weak compared to an allogeneic reaction; nevertheless, the possibility exists that cells early in the T-cell lineage (which might be expected to be present in large numbers in the early neonatal period) are not self-tolerant and express auto-reactivity directed towards antigens which are predominantly expressed on B cells. The crucial prediction of this model is that at least some members of the T-

cell subpopulation we have studied express self-reactivity which can be confirmed in a system other than suppression of antibody synthesis.

These experiments emphasize one practical point for those studying B-cell function in newborn animals; specifically, the suppressor T cells we have studied express very low amounts of the Thy 1 antigen and therefore are difficult to eliminate by using standard protocols for anti-Thy 1 and complement treatment. Unless depletion of suppressor activity is used as a monitor for abolition of T-cell function, we would suggest that experiments viewing the activity of purified neonatal B cells be interpreted with caution.

Summary

Neonatal suppressor T cells were isolated from the thymuses of 10- to 14-day old BDF$_1$ mice infected at birth with mouse thymic virus. Such cells were enriched for suppressive activity directed against antibody formation by adult B cells and represented a relatively homogenous population of outer cortical cells. Their surface antigen phenotype was found to be: Ly 1$^+$, Ly 2$^+$, TL$^+$, Thy 1$^+$ ↑, and H-2$^+$ ↓. The cells were larger and contained more DNA than thymocytes from age-matched controls. These findings identify neonatal suppressor T cells as a unique subpopulation separate from most inducible suppressor cells in the adult mouse. The mechanism of action of neonatal suppressor T cells seems to be a reduction in the number of B cells initially triggered by antigen.

References

1. Mosier, D. E., and B. M. Johnson. 1975. Ontogeny of mouse lymphocyte function. II. Development of the ability to produce antibody is modulated by T lymphocytes. *J. Exp. Med.* 141:216.
2. Kisielow, P., J. A. Hirsch, H. Shiku, P. C. L. Beverley, M. K. Hoffman, E. A. Boyse, and H. F. Oettgen. 1975. Ly antigens as markers for functionally distinct subsets of thymus derived lymphocytes of the mouse. *Nature (Lond.).* 253:219.
3. Shiku, H., P. Kisielow, M. A. Bean, T. Takahashi, E. A. Boyse, H. F. Oettgen, and L. J. Old. 1975. Expression of T-cell differentiation antigens on effector cells in cell mediated cytotoxicity in vitro: evidence for functional heterogeneity related to surface phenotype of T cells. *J. Exp. Med.* 141:227.
4. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of lymphocytes bearing different Ly antigens. I. Generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* 141:1376.
5. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly$^+$ cells in the generation of killer activity. *J. Exp. Med.* 141:1390.
6. Huber, B., O. Devinsky, R. K. Gershon, and H. Cantor. 1976. Cell-mediated immunity: delayed-type hypersensitivity and cytotoxic responses are mediated by different T-cell subclasses. *J. Exp. Med.* 143:1534.

7. Feldman, M., P. C. L. Beverley, M. Dunkley, and S. Kontiainen. 1975. Different Ly antigen phenotypes of *in vitro* induced helper and suppressor cells. *Nature (Lond.)*. 258:614.

8. Beverley, P. C. L., J. Woody, M. Dunkley, M. Feldman, and I. McKenzie. 1976. Separation of suppressor and killer T cells by surface phenotype. *Nature (Lond.)*. 262:495.

9. Cantor, H., F-W. Shen, and E. A. Boyse. 1976. Separation of helper T cells from suppressor T cells expressing different Ly components. II. Activation of antigen: after immunization antigen-specific suppressor and helper activities are mediated by distinct T-cell subclasses. *J. Exp. Med.* 143:1391.

10. Vadas, M. A., J. F. A. P. Miller, I. F. C. McKenzie, S. E. Chism, F-W. Shen, E. A. Boyse, J. R. Gamble, and A. M. Whitelaw. 1976 Ly and Ia antigen phenotypes of T cells involved in delayed-type hypersensitivity and in suppression. *J. Exp. Med.* 144:10.

11. Herzenberg, L. A., K. Okumura, H. Cantor, V. L. Sato, F-W. Shen, and E. A. Boyse. 1976. T-cell regulation of antibody responses: demonstration of allotype-specific helper T cells and their removal by suppressor T cells. *J. Exp. Med.* 144:330.

12. Jandinski, J., H. Cantor, T. Tadakuma, D. L. Peavy, and C. W. Pierce. 1976. Separation of helper T cells from suppressor T-cells expressing different Ly components. I. Polyclonal activation: suppressor and helper activities are inherent properties of distinct T-cell subclasses. *J. Exp. Med.* 143:1382.

13. Weitzman, S., F-W. Shen, and H. Cantor. 1976. Maintenance of hypo-responsiveness to antigen by a distinct subclass of T lymphocytes. *J. Immunol.* 117:2209.

14. Darzynkiewicz, Z., F. Traganos, T. Sharpless, and M. R. Melemed. 1976. Lymphocyte stimulation: A rapid multiparameter analysis. *Proc. Natl. Acad. Sci. U. S. A.* 73:2881.

15. Rowe, W. P., and W. I. Capps. 1961. A new mouse virus causing necrosis of the thymus newborn mice. *J. Exp. Med.* 113:831.

16. Cohen, P. L., S. S. Cross, and D. E. Mosier. 1975. Immunological effects of neonatal infection with mouse thymic virus. *J. Immunol.* 115:706.

17. Cross, S. S., H. C. Morse, III, and R. Asofsky. 1976. Neonatal infection with mouse thymic virus. Differential effects on T cells mediating the graft-versus-host reaction. *J. Immunol.* 117:635.

18. Morse, H. C. III, S. S. Cross, and P. J. Baker. 1976. Neonatal infection with mouse thymic virus: Effects on cells regulating the antibody response to type III pneumococcal polysaccharide. *J. Immunol.* 116:1613.

19. Mosier, D. E., I. Scher, and W. E. Paul. 1976. *In vitro* responses of CBA/N mice: Spleen cells of mice with an X-linked defect that precludes immune responses to several thymus-independent antigens can respond to TNP-lipopolysaccharide. *J. Immunol.* 117:1363.

20. Inman, J. K. 1975. Thymus-independent antigens: the preparation of covalent, hapten-Ficoll conjugates. *J. Immunol.* 114:704.

21. Jacobs, D. M., and D. C. Morrison. 1975. Stimulation of a T-independent primary anti-hapten response *in vitro* by TNP-lipopolysacchardie (TNP-LPS). *J. Immunol.* 114:360.

22. Rittenberg, M. B., and C. Pratt. 1969. Anti-trinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* 132:575.

23. Gorer, P. A., and P. O'Gorman. 1956. The cytoxic activity of iso-antibodies in mice. *Transplant. Bull.* 3:142.

24. Boyse, E. A., L. J. Old, and I. Chouroulinkov. 1964. Cytotoxic test for demonstration of mouse antibody. *Methods Med. Res.* 10:39.

25. Boyse, E. A., L. Hubbard, E. Stockert, and M. E. Lamm. 1970. Improved complementation in the cytotoxic test. *Transplantation*. *(Baltimore)*. 10:446.

26. Shen, F-W., E. A. Boyse, and H. Cantor. 1975. Preparation and use of Ly antisera. Immunogenet. 2:591.

27. Rich, R. R., and C. W. Pierce. 1973. Biological expressions of lymphocyte activation. II. Generation of a population of thymus-derived suppressor lymphocytes. *J. Exp. Med.* 137:649.

28. Scher, I., A. Ahmed, D. M. Strong, A. D. Steinberg, and W. E. Paul. 1975. X-linked B-lymphocyte immune defect in CBA/HN mice. I. Studies of the function and composition of spleen cells. *J. Exp. Med.* 141:788.

29. Scher, I., A. D. Steinberg, A. Berning, and W. E. Paul. 1975. X-linked B-lymphocyte immune defect in DBA/HN mice. II. Studies of the mechanisms underlying the immune defect. *J. Exp. Med.* 142:637.

30. Cohen, P. L., I. Scher, and D. E. Mosier. 1976. *In vitro* studies of the genetically determined unresponsiveness to thymus-independent antigens in CBA/N mice. *J. Immunol.* 116:301.

31. Weissman, I. L., M. Small, C. G. Fathman, and L. A. Herzenberg. 1975. Differentiation of thymus cells. *Fed. Proc.* 34:141.

32. Roelants, G. E., K. S. Mayor, L-B Hägg, and F. Loor. 1976. Immature T lineage lymphocytes in athymic mice. Presence of TL, lifespan and homeostatic regulation. *Eur. J. Immunol.* 6:75.

33. Smith, J. B., and G. J. Eaton. 1976. Suppressor cells in spleens from "nude" mice: Their effect on the mitogenic response of B lymphocytes. *J. Immunol.* 117:319.

34. Howe, M. L., A. L. Goldstein, and J. Battisto. 1970. Isogeneic lymphocyte interaction: Recognition of self-antigens by cells of the neonatal thymus. *Proc. Natl. Acad. Sci. U. S. A.* 67:613.

35. von Boehmer, H., and W. J. Byrd. 1973. Stimulation of mouse thymocytes by syngeneic spleen cells in mixed lymphocyte reactions. *Adv. Exp. Med. Biol.* 29:217.