PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 169, 127–134 (1982)

Tumor-Induced Impairment of T-Cell-Independent B-Cell Blastogenesis and DNA Polymerase Activity[1] (41320)

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Abstract. Fibrosarcoma-bearing BALB/c mouse spleens contained suppressor cells which inhibited B-cell blastogenesis and DNA polymerase activity. Two distinct populations of suppressor cells, macrophages (Mϕ) and T cells, were capable of inhibiting lipopolysaccharide-induced B-cell DNA synthesis and DNA polymerase α activity. Direct suppression of B cells by Mϕ was a function of the *in vitro* concentration of Mϕ employed and inhibition was not mediated via soluble molecules. Suppressor T cells, however, regulated via soluble substances. The results from this study indicated that nonspecific suppressor cells of both the monocyte/Mϕ and T-cell lineage may affect T-cell-independent B-cell blastogenesis and ultimately regulate at the level of DNA replicative enzymes. However, Mϕ may have a minimal inhibitory role in tumor-burdened hosts, at least with respect to T-cell-independent systems.

Many tumor types can induce nonspecific suppressor cells which inhibit T lymphocyte and antibody-forming cellular responses (1–5). The suppressors of antitumor responses have been chiefly identified as tumor-specific T cells; whereas, nonspecific suppressor cells have been ascribed to monocyte/macrophage (Mϕ) lineage (2–5). Recently, we and others have detected in tumor-bearing hosts (TBH) nonspecific suppressor T (T_S) cells capable of inhibiting phytohemagglutinin (PHA) responsiveness (4, 6), contact allergen-induced immune blastogenesis (7, 8), and specific hemolytic plaque formation (5). These experiments demonstrated the presence of two distinct populations of suppressor cells operating independently in the spleens (but not lymph nodes) of fibrosarcoma-bearing BALB/c mice (4, 5). However, these previous studies involved investigations of T-cell-dependent phenomena and did not show the direct effect of suppressor cells on B-cell activities.

DNA polymerases are involved in induced proliferation responses of lymphocytes (9, 10). In an earlier article, we reported depressed DNA polymerase activity in PHA-stimulated TBH T cells (10). These investigations indicated that T_s cells could elaborate soluble factors capable of interacting with DNA polymerase enzymes and inhibiting their activity (8, 10, 11). Similar results were seen using cell sonicates (10). The suppression of B-lymphocyte functions observed in other studies has not been shown definitively to be independent of impaired helper T-cell activity (5). In earlier work, Lemke *et al.* (12), using normal and athymic mouse systems, reported that Mϕ can suppress LPS-induced B-cell blastogenesis directly. The present study was undertaken to determine whether T-cell-independent B-lymphocyte mitogen responses, i.e., lipopolysaccharide responsiveness, are impaired in TBH, what cell is responsible, and whether this impairment is expressed at the level of DNA polymerases.

Materials and Methods. *Animals and tumors.* Male BALB/c mice 6 to 8 weeks old were used in all experiments (Flow Laboratories, Dublin, Va.). Syngeneic fibrosarcomas were initiated by an inoculation of 10^6 viable MBF-1 tumor cells in the left hindleg. MBF-1 is a methylcholanthrene-induced fibrosarcoma initiated and main-

[1] This investigation was supported in part by an Elsa U. Pardee Foundation Grant, by Grant IN 117 from the American Cancer Society, and by a National Institutes of Health Biomedical Research Support Grant.

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0037-9727/82/010127-08$01.00/0

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tained by us. Tumors were palpable by Day 10 postinoculation. Mice with uniform 1-cm-diameter tumors were used throughout.

Lymphoid cell separation. Mice were sacrificed, the spleens, lymph nodes, and thymuses removed aseptically, and cell suspensions prepared by pressing the tissues through No. 50 mesh stainless-steel screens into RPMI 1640 medium (Flow Laboratories, Rockville, Md.). Erythrocytes were lysed by treatment with 0.85% NH_4Cl solution for 5 min and the suspension was washed twice with medium. Cells were resuspended in supplemented culture medium consisting of: (a) RPMI 1640 with 2 m*M* glutamine, (b) penicillin (100 units/ml) and streptomycin (100 μg/ml) (Grand Island Biological Co., Grand Island, N.Y.), (c) 25 m*M* Hepes buffer, (d) 2 g/liter $NaHCO_3$, (e) 4×10^{-5} *M* 2-mercaptoethanol, and (f) 10% heat-inactivated fetal calf serum (4).

Separation of spleen cells into T- and B-cell-enriched populations was accomplished with minor alterations (twice the amount of nylon wool) as described by Julius *et al.* (13). Relatively pure populations of cells could be achieved by these methods, and characterization of the nylon wool-adherent (enriched for B cells) and nonadherent (T cells) fractions has been documented in our earlier studies (4, 15). Briefly, nylon wool-adherent cells were obtained by agitation of the wool in RPMI medium with sterile forceps and aspiration of the released adherent population. The nylon wool-adherent fractions were further enriched for B cells by removing T cells through anti-Thy 1 and complement treatment and Mϕ by two 1-hr platings. T cells were also doubly plated to remove Mϕ. As our previous work showed (4), the T_s cell was moderately adherent to nylon wool, thereby affording us a method for depleting T cells of T_s cells. Macrophages were obtained or depleted from whole spleen cells by plating 10^8 spleen cells on 150-mm plastic culture dishes (Falcon, Becton-Dickinson, Calif.) for 2 hr at 37° (3, 4). Plastic-adherent cells obtained in this manner were then treated with anti-Thy 1 serum and complement as described elsewhere (4, 6, 7, 13) and contained >99% Mϕ as determined by esterase staining. Supernatants were harvested from 48-hr cultures of 2×10^6 cells of the various leukocyte populations, centrifuged at 10,000 *g* for 20 min, and filtered through a 0.45-μm pore-sized filter (4, 6, 7).

Blastogenic assay. Our assay for *in vitro* lymphocyte blastogenesis used [^{3}H]thymidine incorporation into DNA (4). Briefly, a 72-hr microculture (total volume of 0.2 ml) assay system was used to measure blastogenesis after stimulation with 10 μg LPS (*Escherichia coli* lipopolysaccharide 026: B6, Sigma Chemical Co., St. Louis, Mo.) per culture well. All cultures received 2×10^5 cells and, in some instances, 10^5 TBH spleen cells, or various percentages of Mϕ which were admixed to detect suppressor cell activity. Appropriate normal filler cell controls indicated that inhibition was not an artifact due to an *in vitro* crowding effect. Experiments conducted to determine the extent of thymidine participation in the assay showed that the presence of "cold thymidine" could not account for inhibition of blastogenesis (14). Eighteen hours before assay termination, the cultures were pulsed with 1 μCi of [^{3}H]thymidine ([^{3}H]TdR; sp act, 1.9 Ci/mmole; Schwarzmann, Orangeburg, N.Y.). Cultures were harvested by a Multiple Automated Sample Harvester II (Microbiological Associates, Bethesda, Md.) and counted by liquid scintillation spectrometry. Mitogen-induced DNA synthesis was expressed as the difference between the means of unstimulated and stimulated (LPS) cultures as measured by counts per minute (cpm) (4).

Lymphoid polymerase preparation and DNA polymerase assay. Crude DNA polymerase fractions were prepared by sonication and clarification of spleen cells in isotonic buffer as previously described (10). Sonicates of Mϕ-depleted anti-Thy 1-treated nylon wool adherent spleen cells contained DNA polymerase α, β, and γ activities (4).

DNA polymerase activity was measured by the incorporation of radioactively labeled substrate ([^{3}H]dTTP; sp act, 24.5 Ci/mmole; Amersham Corp., Arlington Heights, Ill.) into membrane-bound poly-

merized product as measured by liquid scintillation spectrometry. The three DNA polymerase (α, β, and γ) activities (15–18) may be selectively assessed by mixing lymphocyte sonicates 1:1 (v/v) with reaction mixtures that utilize templates, salt concentrations, and inhibitors to allow maximal activity of only one enzyme. The reaction mixture used to measure DNA polymerase α, the replicative enzyme most active in S phase, consisted of: 20 m*M* KH_2PO_4 (pH 7.8), 0.2 m*M* each dATP, dCTP, dGTP, and [^{3}H]dTTP (80 cpm/pmole), 7.5 m*M* $MgCl_2$, 50 μg DNase-activated calf thymus DNA (16), 0.5 m*M* dithiothreitol, and 50 μl of polymerase preparation in a final volume of 110 μl. Reaction mixtures to measure polymerase β, the suggested repair enzyme (9, 16), consisted of: 50 m*M* Tris–HCl (pH 8.5), 0.2 m*M* each dATP, dCTP, dGTP, and [^{3}H]dTTP, 7.5 m*M* $MgCl_2$, 50 μg activated calf thymus DNA, 0.5 m*M* dithiothreitol, 0.2 *M* NaCl, 10 m*M* *N*-ethylmalemide (a specific inhibitor of DNA polymerase α and γ) and 50 μl of polymerase extract. Polymerase γ, which specifically polymerized dTTP with poly(rA):oligo(dT) templates (19, 20), was measured by a mixture which consisted of: 50 m*M* Tris–HCl (pH 7.5), 0.2 m*M* [^{3}H]dTTP, 0.5 m*M* $MnCl_2$, 2.5 μg poly(rA):oligo(dT), 0.5 m*M* dithiothreitol, 0.1 *M* KCl, and 50 μl of the crude polymerase preparation.

Under normal assay conditions, polymerase reactions were run for 2 hr at 37°C. One hundred-microliter aliquots were then removed, placed onto Whatman DE81 filters, and washed with 0.1 *M* sodium pyrophosphate, 0.3 *M* ammonium formate, pH 7.8, and 95% ethanol. Filters were dried with an infrared lamp and counted by liquid scintillation spectrometry. Picomoles of [^{3}H]dTTP incorporated was determined by dividing filter counts per minute by a predetermined value of 80 cpm/pmole [^{3}H] dTTP. For further details of the polymerase assays see our previous publication (10).

Results. *LPS-induced blastogenesis.* Normal and TBH spleen cells were stimulated with LPS, and DNA synthesis was measured by [^{3}H]TdR incorporation (Table I). The normal spleen cell data demonstrated the selective enrichment for B cells in the nylon wool-adherent fraction by consistently higher LPS stimulation values for the population. Further characterization of the nylon wool-adherent fraction is reported elsewhere (4). Nonadherent cells did not respond to LPS (4). When compared to normal stimulation values, both TBH whole spleen cells and nylon wool-adherent B cells were highly suppressed. The suppression seen in the nylon wool-adherent fraction indicated that a suppressor cell, as shown in an earlier study (4), can be separated by nylon wool because it is adherent.

Effects of Mφ on B-cell blastogenesis. Titration of normal or TBH anti-Thy 1-treated splenic Mφ demonstrated progressive inhibition of LPS-induced blastogen-

TABLE I. DNA SYNTHESIS IN MURINE SPLENIC LYMPHOCYTES

Cell source	DNA synthesis [^{3}H]TdR incorporation (cpm)[a]	
	Stimulant: None	LPS
Whole spleen cells[b]		
Normal	1734 ± 204	12,682 ± 2600
TBH[c]	2247 ± 186	2,826 ± 800
B-cell-enriched, nylon wool adherent population		
Normal	2370 ± 217	26,927 ± 2015
TBH[c]	4509 ± 158	6,735 ± 650

[a] Results are expressed as mean cpm ± SE of cells from six pooled spleens in quadruplicate cultures from a representative experiment.

[b] Cultures consisted of 2 × 10^5 cells/well stimulated with an optimum dose of mitogen.

[c] TBH spleen cells responded maximally at the same optimal normal cell dosage of LPS as determined by titration (not shown).

esis. The inhibition of normal B cells was dependent on the ratio of Mφ to LPS-responsive cells and there was no significant difference between the values for normal or TBH Mφ. Adding Mφ to LPS-responsive cells in the amounts of 0, 10, and 50%, resulted in [^{3}H]TdR uptake of approximately 55,000, 48,000, and 7000 cpm, respectively (data not shown). This clearly indicates that the greater the *in vitro* concentration of T-cell-depleted Mφ, the more dramatic the suppression of B-cell responsiveness.

TBH splenic suppressor cells. The B-cell-enriched, nylon wool adherent spleen cells which had been treated with anti-Thy 1 and plated twice (i.e., B cells) were admixed with TBH whole spleen cells and nylon wool separated spleen cells (Table II). B-cell stimulation with LPS was significantly inhibited by TBH whole spleen cells (40%) or the B-cell-enriched, nylon wool-adherent spleen cell population (84%). T cells did not contain a significant number of cells suppressive for LPS stimulation and did not respond to LPS. To discover whether Mφ or T_s cells were responsible for the observed inhibition, TBH spleen cells were either depleted of Mφ by successive tissue culture dish platings which reduced Mφ contamination to levels of $<0.5\%$ (4, 15) or treated with anti-Thy 1, or both. Macrophage depletion of TBH spleen cells did not alleviate inhibition; however, suppression was sensitive to anti-Thy 1 treatment, suggesting the presence of T_s cells. The lack in reduction of suppression by Mφ-depleted TBH whole spleen cells may imply a requirement for a larger number of Mφ. Furthermore, when the enriched B-cell, T_s-cell, and Mφ population (nylon wool-adherent fraction) was depleted of Mφ there was an 11% reduction in suppression (significantly different at $p = 0.05$). Although the contribution of Mφ to the direct suppression of TBH T-cell-independent B-cell proliferation may be minimal, this is not so in T-cell-dependent systems (1–5).

Mediation of suppression via soluble factors. We have reported previously that culture supernatants from tumor-induced suppressor cells inhibited normal spleen cell PHA responsiveness (4). The next experiment was designed to determine whether Mφ and/or T_s cells elaborated soluble substances that could also impair LPS responses of normal B cells. As can be seen from the data in Table III, supernatants from TBH whole spleen cells, nylon wool adherent cells (B cell enriched but not depleted of T_s cells or Mφ), and thymocytes all contained material inhibitory for LPS responsiveness. Anti-Thy 1 treatment of TBH spleen cells before culturing removed the suppressor cells responsible for the nonspecific inhibitory factor(s). Neither normal nor TBH anti-Thy 1-treated Mφ elaborated any suppressive substances.

LPS-induced DNA polymerase activity. DNA polymerase activity of unstimulated and LPS stimulated spleen cell cultures was compared between normal and TBH (Table IV). Unstimulated TBH whole spleen cells and B cells demonstrated elevated levels of polymerase α activity when compared to their normal counterparts. Upon the addition of mitogen to cultures, normal spleen cell and B-cell-enriched, nylon wool-adherent spleen cell DNA polymerase activity increased significantly which corresponded with increased DNA synthesis (Table I). There were marginal increases in DNA polymerase β and γ activity. TBH whole spleen cells and the B-cell-enriched population, when stimulated with LPS, showed significant inhibition of DNA polymerase α activity below basal levels. DNA polymerase β and γ were not significantly affected. The suppression observed is not unique to LPS since we have reported it for PHA (10) and antigen (8). These results imply an indirect effect of mitogen stimulation on DNA polymerase α and that the biological regulation of blastogenesis mediated through suppressor cells and/or their products may also be expressed at the molecular level of DNA replicative enzymes.

Discussion. Results presented in this paper illustrated tumor-induced suppression of T-cell-independent B-cell blastogenesis. Inhibition was due to the combined (but independently suppressive) regulatory influence of two distinct suppressor cells. Macrophages were directly suppressive to B-cell blastogenesis in a system containing

TABLE II. SUPPRESSOR CELL ACTIVITY IN TBH SPLEENS

Composition of culture: 2×10^5 normal cells[a]	+ 10^5 TBH cells[b]	Treatment of admixed TBH cells[c]	[^{3}H]TdR incorporation[d]: None	[^{3}H]TdR incorporation[d]: LPS	Percentage inhibition[e]
Whole spleen cells	—		3103 ± 161	16,402 ± 1310	
B cells	—		4193 ± 145	32,183 ± 1562	
T cells	—		1417 ± 113	1,508 ± 94	
—	Whole spleen cells		2153 ± 212	6,065 ± 314	
—	B-Cell-enriched, nylon wool adherent spleen cell population		2337 ± 283	2,062 ± 203	
—	T cells[f]		2146 ± 406	2,087 ± 391	
B cells	Whole spleen cells	None	2877 ± 83	19,782 ± 960	40
B cells	B-Cell-enriched, nylon wool adherent spleen cell population	None	3548 ± 223	8,113 ± 617	84
B cells	T cells	None	5665 ± 329	30,397 ± 1606	12
B cells	Whole spleen cells	Mϕ-depleted	2163 ± 203	17,816 ± 549	44
B cells	B-Cell-enriched, nylon wool adherent spleen cell population	Mϕ-depleted	4082 ± 141	11,734 ± 158	73
B cells	T cells	Mϕ-depleted	3876 ± 382	32,887 ± 2037	0
B cells	Whole spleen cells	Mϕ-depleted plus anti-Thy 1	3452 ± 198	28,998 ± 1871	8
B cells	B-Cell-enriched, nylon wool adherent spleen cell population	Mϕ-depleted plus anti-Thy 1	2866 ± 415	33,061 ± 1443	0

[a] Normal B cells were obtained from nylon wool-adherent fractions that were anti-Thy 1-treated and plated twice. Normal T cells were nylon wool nonadherent and doubly plated to remove Mϕ.

[b] Tumor-bearing mouse B and T cells (admixed cells) were initially purified by nylon wool columns, then either Mϕ-depleted or Mϕ-depleted plus anti-Thy 1-treated (see footnote *c*).

[c] Before admixture TBH whole spleen cells, B-cell-enriched, nylon wool adherent-spleen cells (B cells), or T cells were exposed to either no treatment (none), Mϕ removal (Mϕ-depleted) or Mϕ removal plus anti-Thy 1 (Mϕ-depleted plus anti-Thy 1). Macrophage depletion consisted of two successive 1-hr plastic culture dish incubations which reduced Mϕ contamination to $<0.5\%$ as determined by esterase staining.

[d] Results are expressed as mean cpm ± SE of quadruplicate cultures of a representive experiment.

[e] Percentage of inhibition was determined by the following formula: [(control − test)/control] × 100.

[f] T cells were collected (only instance) from a standard (allows mildly adherent T_s cells through) nylon wool column.

TABLE III. TBH LEUKOCYTE CULTURE SUPERNATANT INHIBITION OF B-CELL BLASTOGENESIS

Supernatant source (2×10^6 cells/ml)[a]	$[^3H]$TdR incorporation[b]		Percentage inhibition[c]
	None	LPS	
None	5213 ± 227	48,763 ± 3849	
Normal whole spleen cells	6483 ± 129	51,906 ± 3105	
Normal Mφ	6310 ± 267	53,116 ± 1291	
Tumor-bearing mice			
Whole spleen cells	4482 ± 281	18,337 ± 476	70
B-Cell-enriched, nylon wool adherent cell population	3651 ± 473	12,842 ± 1399	80
Whole spleen cells + anti-Thy 1[d]	4763 ± 444	46,277 ± 2768	0
B-Cell-enriched, nylon wool adherent cell population + anti-Thy 1[d]	4228 ± 394	50,692 ± 4002	0
T cells	5213 ± 568	48,274 ± 2887	0
Thymus cells[e]	4781 ± 208	12,176 ± 1136	84
Lymph node cells	5456 ± 142	55,489 ± 1061	0
TBH Mφ	7118 ± 765	49,689 ± 2782	9

[a] Cells were cultured for 48 hr in 35-mm culture plates. Supernatants were collected, centrifuged, and passed through a 0.45-μm Millipore filter. Normal whole spleen cell culture supernatants served as the control. All supernatants were tested against 2×10^5 normal B cells which were obtained as described in Table II. Supernatant-treated cultures demonstrated no difference in viability when compared to untreated cultures, as determined by trypan blue exclusion.

[b] Results are expressed as mean cpm ± SE of quadruplicate cultures.

[c] Percentage of inhibition was calculated as described in Table II.

[d] Preincubated with anti-Thy 1 plus complement.

[e] Supernatants from normal thymus cells had no suppressive activity.

only B cells and Mφ plus mitogen. Macrophage suppression of LPS-induced blastogenesis in T-cell and Mφ-depleted splenic B cells was directly related to the *in vitro* concentration of Mφ employed. In this tumor model, spleens may contain up to 25% Mφ. However, results suggest that Mφ may have only a marginal influence on *in vivo* T-cell-independent B-cell blastogenesis. It has been reported that the high *in vivo* splenic Mφ concentration may also cause impaired T-cell-mediated responses (4, 6).

No functional difference was detected between normal and TBH Mφ in their ability to modulate LPS-induced blastogenesis. Our inability to detect any soluble inhibitory effects of Mφ on LPS stimulation may indicate a contact requirement for suppression as suggested by Baird and Kaplan (21).

Suppressor T cells also had a negative regulatory influence on B-cell LPS responsiveness. Macrophage-depleted TBH spleen cells revealed a mildly nylon wool-adherent anti-Thy 1 sensitive cell which impaired both B-cell LPS-stimulated and, previously reported, PHA-stimulated responses (4). In contrast with Mφ inhibition, T_s-cell effects were achieved via a soluble factor(s).

Inhibition of polyclonal expansion seems to be at least one mechanism by which regulatory cells may operate with resulting nonspecific suppression of the immune response. Suppressor cells, used in this tumor model, have also been shown to inhibit T-cell-dependent mixed-lymphocyte reactions (15, 22) and immune blastogenesis in contact-sensitized mice (7, 8, 23). Unstimulated DNA synthesis in TBH spleen cells was consistently higher than their normal counterparts (Table I) and DNA polymerase α activity was also elevated in these cells (Table IV). Upon the addition of mitogen to TBH spleen cells, however, there was negligible stimulation of DNA synthesis and DNA polymerase α activity fell below unstimulated cultures. Had DNA polymerase α activity remained unchanged, suppression of DNA synthesis might be attributed to a mechanism involving interference with mitogen stimulation. The data showed this not to be the case. Mitogen stimulation may act as a "second" signal

TABLE IV. DNA POLYMERASE ACTIVITY OF MITOGEN-INDUCED SPLENIC LYMPHOCYTES

Cell Source[a]	Stimulant[b]: None			LPS		
	pmol [³H]dTTP incorporated × 10²					
	α	β	γ	α	β	γ
Whole spleen cells						
Normal	26.9 ± 0.07	13.5 ± 0.09	10.6 ± 0.10	35.5 ± 0.09	15.4 ± 0.02	11.0 ± 0.05
TBH	93.0 ± 0.10	14.6 ± 0.12	12.1 ± 0.09	31.6 ± 0.03	14.8 ± 0.08	12.0 ± 0.07
B-Cell-enriched nylon wool-adherent spleen cells						
Normal	40.1 ± 0.06	14.4 ± 0.09	10.2 ± 0.05	83.6 ± 0.04	19.4 ± 0.12	11.6 ± 0.13
TBH	58.3 ± 0.03	14.0 ± 0.04	11.4 ± 0.12	31.9 ± 0.08	13.7 ± 0.08	11.7 ± 0.09

[a] B cells were not treated with anti-Thy 1 or Mϕ-depleted, therefore, represent a population enriched for B cells and contaminated with T_s cells and Mϕ. TBH B cells free of T_s cells and high numbers of Mϕ respond normally.

[b] Cells were incubated either without or with an optimum dosage of LPS (50 μg LPS/10^6 cells) for 72 hr. All cells tested in this experiment were diluted to 2×10^7 cells and then sonicated for preparation of polymerase extracts. The amount of enzyme activity is per 2×10^7 cells.

for the pronounced inhibition of DNA polymerase α activity in TBH spleen cells (Table IV).

In an earlier study, we demonstrated that one of the regulatory influences of tumor-induced T_s cells was the elaboration of a soluble factor(s) that inhibited DNA synthesis in PHA-stimulated syngeneic spleen cells and other mammalian cell lines (10). It was further shown that T_s-cell-derived soluble molecules could inhibit DNA synthesis *in vitro*, thus representing one mechanism by which T_s cells may operate. Other investigators have also reported the direct interaction of PHA-induced suppressor cell products with purified DNA polymerase α (11). We recently reported on the purification of factors which inhibit DNA polymerase α (IDP) and DNA synthesis (IDS) (24).

The results of these earlier studies and the data herein strongly suggest that tumor-induced T_s cell and perhaps Mϕ regulation may be manifested in both T-cell-dependent and -independent blastogenic cellular events. This could also imply that tumor-induced suppression may operate directly on B cells, i.e., on antibody forming cells, without the impairment of an intermediary regulatory helper T cell. The necessity of stimulation may mean a requirement for an altered cell cycle, membrane changes, or the direct interaction of suppressive substances with the stimulant. However, the ultimate point of proliferative control may be the limitation of the DNA polymerase α enzyme.

The authors wish to thank Dr. Alan M. Kaplan for his review of the manuscript and Ms. Connie Melton for her excellent secretarial help.

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Received March 3, 1981. P.S.E.B.M. 1982, Vol. 169.