

MECHANISM OF SUPPRESSION OF
LIPOPOLYSACCHARIDE-DRIVEN B CELL
DIFFERENTIATION BY ANTI- μ ANTIBODIES
Evidence for a *Trans*-acting Repressor of Transcription

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Crosslinking of the IgM receptor of B lymphocytes by bivalent antibodies can initiate cell enlargement and receptiveness to growth factors that trigger entry into the growth cycle. In the presence of other T cell-derived factors, some B lymphocytes activated in this manner differentiate into antibody-secreting cells (1). Anti- μ chain antibodies, usually at higher concentrations than are optimal for B cell activation, can also inhibit B cell differentiation. The most widely studied system has used bacterial LPS as a polyclonal activator of murine B cells (2, 3) but similar effects have been demonstrated with human B cells triggered by the T-dependent activator PWM.

Differentiation of resting B cells to antibody-secreting cells generally requires one or more rounds of cell division. Anti- μ antibodies selectively inhibit differentiation of LPS-stimulated B cells while enhancing their proliferation. >95% of cells from 4–5-d suppressed cultures are Ia⁺ and will rapidly reexpress surface IgM (sIgM)¹ on removal of the antibody, but few, if any, are capable of differentiating into antibody-secreting cells (4).

The LPS system has been particularly useful for studies of differentiation at the molecular level. After LPS activation there is a selective increase in transcription of the VDJ-C μ -C δ complex associated with a shift from production of roughly equal quantities of mRNAs for secreted (μ s) and membrane (μ m) chains to a large predominance of μ s (5). The transcription of mRNA for J chain is coordinately enhanced (6). Several groups have recently reported (7–9) the effects of anti- μ suppression on differentiation-related gene activation. In suppressed cultures there is a striking reduction in levels of mRNAs for μ chain, κ chain, and J chain as compared with controls, whereas levels of mRNAs for class I and class II MHC molecules and actin are not influenced. In one study (8), the decrease in μ mRNA was shown to be at least partially accounted for by decreased transcription.

It is clear from these experiments that the inhibition of transcription is not immunologically specific, but is highly selective for differentiation-related genes. We now report observations on the induction of the suppressive signal. We show that suppression does not require internalization of the sIgM–anti- μ complexes

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¹ *Abbreviations used in this paper:* cIgM, cytoplasmic IgM; μ m, membrane μ chain RNA; μ s, secreted μ chain RNA; sIgM, surface IgM.

formed on the cell surface. Induction of suppression by pulse treatment with anti- μ at culture initiation is inhibited by drugs that prevent mRNA or protein synthesis. These results suggest that suppression is mediated by *trans*-acting repressor molecules that modulate transcription of a family of differentiation-related genes.

Materials and Methods

Animals. Adult female C57BL/6 mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were maintained and killed according to National Institutes of Health (Bethesda, MD) guidelines.

Antibodies. Goat antiserum to μ chains and rhodamine-conjugated anti- μ antibodies were obtained from Southern Biotechnology Associates, Inc., Birmingham, AL. Purified anti- μ antibodies were prepared by passage over immunoabsorbent columns as described (3). Anti- μ was covalently linked to agarose beads (Bio-Gel A 1.5M; Bio-Rad Laboratories, Richmond, CA) activated with cyanogen bromide at a ratio of 2 mg anti- μ per milliliter of packed beads (10). Coupling was assumed to be complete. F(ab')₂ fragments were prepared by pepsin digestion and purified by passage over a Sephadex G-150 column. Fluorescent staining for determination of cytoplasmic IgM-bearing cells was done on cytocentrifuge preparations as described (3).

Metabolic Inhibitors. Cordycepin, emetine, cytochalasin B, and colchicine were obtained from Sigma Chemical Co., St. Louis, MO. α -amanitin was supplied by Boehringer Mannheim Biochemicals, Indianapolis, IN.

Cells and Culture. Single-cell spleen suspensions were generated by pressing the spleens between the ground glass ends of microscope slides. Mononuclear cells were recovered by centrifugation of the cell suspensions over a cushion of Ficoll-Hypaque, $\rho = 1.09$. After washing, cells were suspended in culture medium consisting of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Gibco Laboratories), 2 mM glutamine, 50 μ M 2-ME, 10 mM Hepes, pH 7.4, and 50 μ g/ml gentamicin (complete medium).

Cell aliquots were loaded with different concentrations of drug for a period of 30 min; in experiments with α -amanitin the loading period was extended to 1 h because the drug is taken up slowly. LPS and anti- μ were then added, each at a concentration of 50 μ g/ml, and the cells were cultured for 18–24 h at a density of 5×10^5 cells/ml and 1 ml/5-cm² surface area in 24-well plates (Costar, Cambridge, MA). Cells were harvested, washed twice in RPMI with 2% FCS and 10 mM Hepes, pH 7.4, and placed back in culture at the same density in complete medium containing 50 μ g/ml LPS for an additional 4 d. All cultures were maintained in a humidified atmosphere of 5% CO₂ and air at 37°C.

Analysis of μ Chain RNA. Total cellular RNA was extracted and 10- μ g quantities were analyzed by Northern blot with a C μ probe as described previously (9). Autoradiographs were scanned using a densitometer (model 4310; Ortec, Oak Ridge, TN) to determine relative quantities of mRNA.

Statistical Analysis. Paired-difference analysis of percent cytoplasmic IgM (cIgM) expression from each experiment was used to determine significance. For experiments using inhibitors, comparisons were made for the difference between anti- μ pulse-suppressed cells and anti- μ pulse-suppressed cells receiving a metabolic inhibitor during the pulse phase. Statistical tests were performed using Student's *t* test for paired differences.

Results

Initial experiments established that significant suppression of the 5-d plasma cell response to LPS could be achieved by pulsing with anti- μ at culture initiation (4). Because overnight (18–24 h) treatment with anti- μ suppressed the response by >70% in nearly all experiments (data not shown), this period was used in experiments investigating the effects of several metabolic inhibitors upon the induction of suppression.

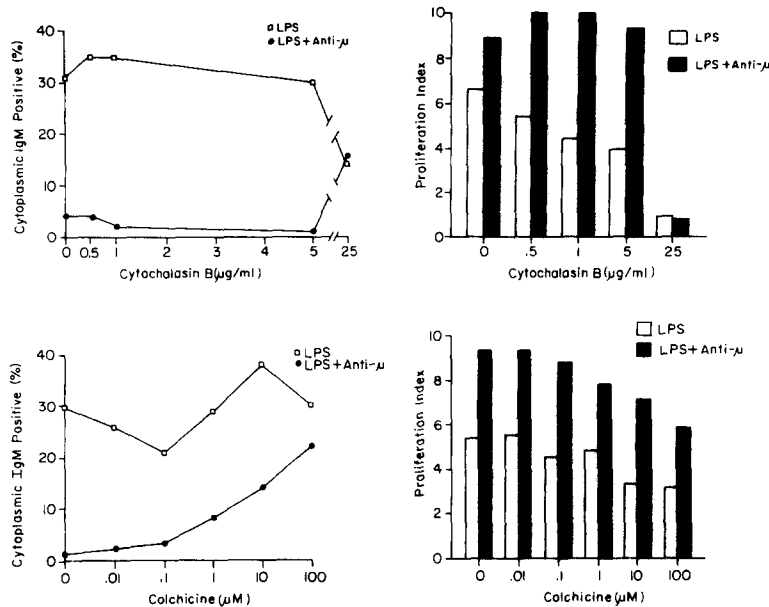


FIGURE 1. Effects of inhibitors of microtubule and microfilament assembly on anti- μ -induced suppression. Splenic lymphocytes were cultured with LPS or LPS plus F(ab')₂ anti- μ with inhibitors at the concentrations shown for 18 h, washed twice, and returned to culture with LPS for an additional 4 d. Recovered cell numbers and the percentage of cells expressing cIgM were then determined. The panels on the left show the percent of cells containing cIgM, while the panels on the right show the numbers of cells harvested from each culture. *Open squares and bars*, LPS cultures; *closed circles and bars*, LPS + anti- μ cultures.

The general design of the experiments was to load cells with a drug, add LPS or LPS and anti- μ antibodies for a period of 18–24 h, wash out drug and antibody, and then culture the cells for an additional 4-d period in complete medium containing LPS. Interference with the induction of suppression was then measured by an increase in the plasma cell response in drug-treated cultures. The enhanced proliferation of anti- μ -treated cultures provided a convenient index of nonspecific drug toxicity. We accepted for analysis only experiments in which the number of cells recovered from cultures containing anti- μ and a given concentration of drug was more than the number recovered from the control cultures treated with LPS alone. In addition, we eliminated as unevaluable a few experiments in which the plasma cell response of the anti- μ -treated control was >30% of the LPS control. The failures of suppression were largely attributable to an unusually low plasma cell response in the LPS control.

Fig. 1 shows an experiment in which cells were treated with varying concentrations of cytochalasin B or colchicine during the pulse with anti- μ . Cytochalasin B inhibits actin polymerization and results in microfilament disruption (11). Microfilaments are involved in maintaining intracellular architecture and the movement of proteins in the membranes and within the cell. Colchicine prevents microtubule assembly in the cell, altering cell architecture and interfering with internal transport systems (12).

At concentrations up to 5 μ g/ml, cytochalasin B neither reduced the plasma

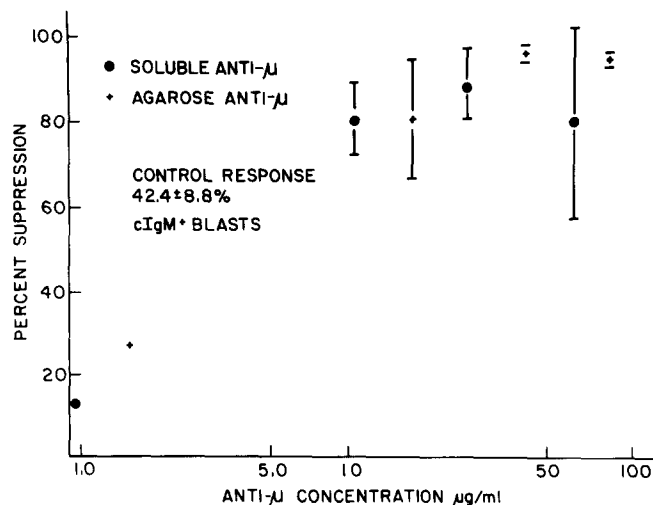


FIGURE 2. Effects of soluble anti- μ and immobilized anti- μ on B cell differentiation. Splenic lymphocytes stimulated with LPS were cultured with soluble anti- μ or anti- μ coupled to Sepharose beads for 5 d and stained for cIgM expression. Results are expressed as the average percent suppression \pm SEM vs. the LPS control for three experiments. *Solid circles*, soluble anti- μ ; *crosses*, agarose-bound anti- μ .

cell response to LPS nor interfered with the induction of suppression by anti- μ . At 25 $\mu\text{g/ml}$ there was a sharp fall in the control response and a rise in the response of the anti- μ -treated culture. As the cell yields fell dramatically at this concentration, we consider the apparent relief of suppression uninterpretable.

The results obtained with colchicine were quite different. In the absence of drug, anti- μ suppressed the LPS response by $\sim 90\%$. At concentrations between 0.1 and 100 μM there was a striking dose-related increase in the response of the anti- μ -treated cells to levels very close to those achieved in the LPS-treated controls. Since at the highest concentration of colchicine used the yield of cells from anti- μ -treated cultures was sixfold greater than input numbers, the data suggest that colchicine interferes with suppression-induction in a specific manner.

The failure of cytochalasin B to interfere with suppression suggested that endocytosis of the anti- μ -IgM receptor complex is not required for suppression induction. In a previous study (4) the observation that B cells from anti- μ -treated cultures contained accumulations of goat anti- μ in the Golgi complex had led to the opposite conclusion. To resolve this issue we compared the effects of anti- μ covalently attached to agarose beads with soluble anti- μ on the response to LPS. Pooled results from three experiments are shown in Fig. 2. Agarose-bound anti- μ is as effective as soluble antibody in inducing suppression. This result is consistent with the failure of cytochalasin B to interfere with suppression.

Inhibitors of mRNA synthesis and protein translation also interfered with suppression of differentiation by anti- μ antibodies. Fig. 3 depicts results from experiments using α -amanitin, cordycepin, and emetine. α -amanitin specifically inhibits transcription of new messenger RNA by binding to RNA polymerase II in the nucleus. Initiation of transcription occurs normally, but elongation of nascent RNA chains is prevented (13). Polymerases I and III are not affected by α -amanitin at the concentrations used in these experiments. Cordycepin (3'-deoxyadenosine) blocks polyadenylation of newly transcribed RNA (14-16). In the presence of cordycepin, messenger RNAs are not transported to the cytoplasm presumably because of the absence of the poly(A) adduct. As with α -

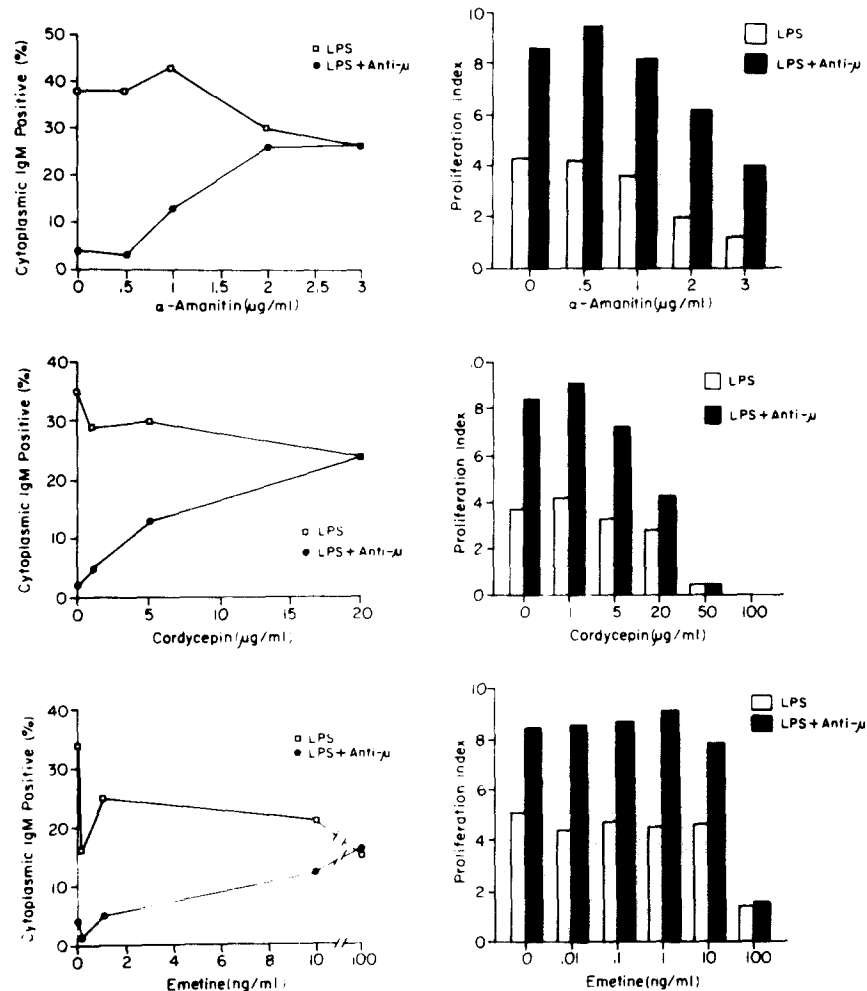


FIGURE 3. Effects of inhibitors of transcription and translation on anti- μ suppression. Cells and treatment were as described in Fig. 1. The panels on the left show the percent of cells containing cIgM, while the panels on the right show the numbers of cells harvested from each culture. Open squares and bars, LPS cultures; closed circles and bars, LPS + anti- μ cultures.

amanitin, new mRNAs do not leave the nucleus, but the site of action of these two inhibitors is entirely different. Emetine is a highly specific inhibitor of eukaryotic ribosomal translocation along mRNA and inhibits new protein synthesis in the cytoplasm (17).

The presence of α -amanitin during the pulse had little effect on cIgM expression in the LPS-stimulated control. Expression was reduced by nearly 90% after an anti- μ pulse, but was restored if 2 μ g/ml α -amanitin was present during the pulse. Thus α -amanitin blocked induction of suppression by anti- μ antibodies without interfering with cell growth.

Cordycepin concentrations from 1 to 50 μ g/ml interfered with suppression induction by anti- μ in a dose-related fashion. Augmented cell proliferation was detected at concentrations up to 20 μ g/ml. Cordycepin was toxic at 100 μ g/ml

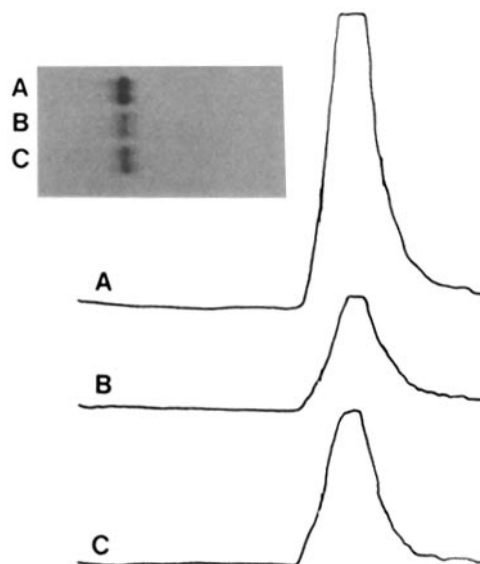


FIGURE 4. Expression of μ chain RNA by cells pulsed with anti- μ in the presence or absence of α -amanitin. Cells were cultured as described in Fig. 1. 10 μ g of whole-cell RNA was electrophoresed in each lane and hybridized to a $C\mu$ probe. Densitometric scans of autoradiograph of Northern blot are shown. (A) LPS control culture; (B) LPS culture pulsed with anti- μ for 18 h; (C) anti- μ pulse in the presence of 2 μ g/ml α -amanitin.

and no cells were recovered, while at 50 μ g/ml cell recovery was less than at the start of culture. Expression of cIgM in LPS-stimulated control cultures was only slightly decreased by cordycepin at concentrations up to 20 μ g/ml.

At concentrations up to 10 ng/ml, emetine had little effect on LPS control cells, while suppression induction, but not growth stimulation, of anti- μ -treated cells was inhibited in a dose-dependent manner.

Analyses of mRNA levels for μ s chain from an experiment with α -amanitin are shown in Fig. 4. The frequency of cIgM⁺ cells in the LPS control culture was 24%, compared with 2% for the anti- μ -pulsed culture and 17% for the anti- μ plus 2 μ g/ml α -amanitin culture. At the completion of incubation, the frequency of sIgM⁺ cells for LPS control, anti- μ -pulsed, and anti- μ plus α -amanitin-pulsed cultures were 83, 92, and 87%, respectively. The autoradiograph obtained from a Northern blot of whole cell RNA from each culture was scanned with a densitometer and tracings of the scans were made to create the figure. To quantitate relative mRNA levels of the cultures, the area under the curves was determined by integration using the areas between the parallel lines to eliminate effects from the shoulders. The relative μ chain RNA levels are: LPS control, 1; anti- μ -pulsed, 0.35; anti- μ plus α -amanitin-pulsed, 0.49.

Pooled results for cIgM expression from several experiments with α -amanitin, cordycepin, emetine, and colchicine are illustrated in Fig. 5. In each case, as the concentration of inhibitor present during the anti- μ pulse was increased, the antibody-mediated suppression decreased. The changes were significant at the 95% confidence level for one or more concentrations of each of the four drugs, as shown in Table I.

Discussion

These studies indicate that suppression of LPS-driven differentiation by anti- μ antibodies is an active process requiring both the transcription and translation

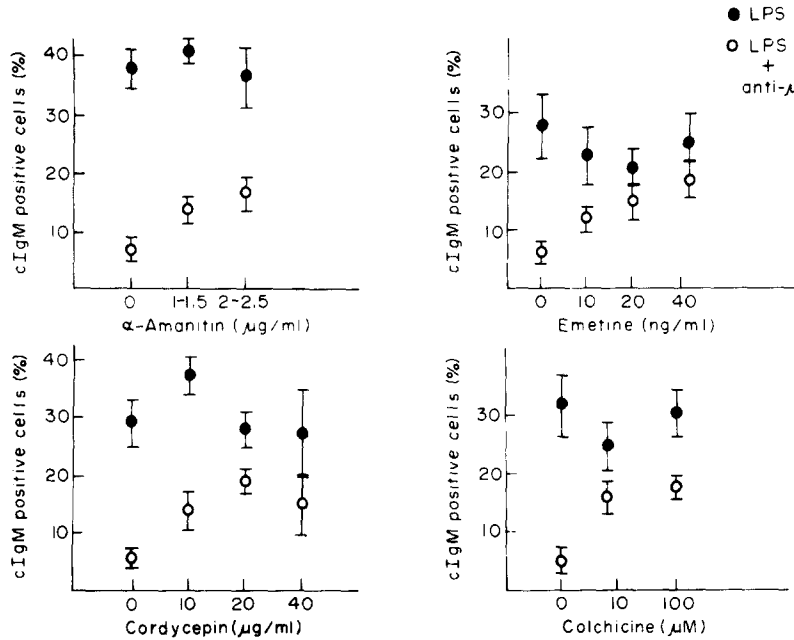


FIGURE 5. Summary of results from trials with inhibitors of RNA, protein, and microtubule synthesis. Each point represents an average of several experiments bracketed by bars depicting the standard error of the mean. The number of observations per point are listed in Table I. Closed circles, LPS controls; open circles, anti- μ -pulsed cells.

TABLE I
Cytoplasmic IgM Expression in LPS-stimulated cultures treated with anti- μ plus Metabolic Inhibitor vs. Anti- μ Control: Statistical Analysis of Paired Differences

Statistical analysis*	IgM expression									
	Amanitin ($\mu\text{g/ml}$) [‡]		Cordycepin ($\mu\text{g/ml}$)			Emetine (ng/ml)			Colchicine (μM)	
	1-1.5	2-2.5	10	20	40	10	20	40	10	100
Difference [§]	5.3 ± 1.9	10.6 ± 3.4	6.5 ± 2.6	14.0 ± 2.6	11.7 ± 4.2	6.17 ± 1.2	11.0 ± 2.1	12.0 ± 2.7	10.6 ± 2.5	11.3 ± 4.8
n	3	5	4	6	3	6	4	4	5	3
s	3.3215	7.668	5.26	6.387	7.234	2.858	4.243	5.477	5.639	8.368
t	2.874	3.091	2.471	5.369	2.794	5.288	5.185	4.382	4.203	2.334
p	0.051	0.018	0.045	0.001	0.054	0.002	0.007	0.011	0.007	0.072

* Symbols used: n, number of pairs; s, standard deviation of difference; t, t statistic; p, probability.

[‡] To obtain sufficient points for analysis, data from 1 to 1.5 $\mu\text{g/ml}$ and 2 to 2.5 $\mu\text{g/ml}$ were combined.

[§] Average of paired differences \pm SEM between drug-treated and control cultures.

^{||} Not every concentration of drug was used for each experiment; the number of pairs per concentration varies.

of one or more new mRNAs. Daughter cells produced in culture have limited ability to differentiate in the presence of LPS even after anti- μ is removed, suggesting that the suppressive factor may be transmitted to the daughter cells. Anti- μ suppression results in inhibition of transcription of a family of genes coordinately expressed after stimulation of B cells by LPS (7-9). Activation of a *trans*-acting repressor system recognizing unique regulatory sequences shared by this functional gene family could explain the results described here. A similar requirement for RNA and protein synthesis in the induction of tolerance among adult (18) and neonatal (19) B cells has been previously demonstrated.

Transcriptional regulation of Ig gene expression is lymphoid cell specific and involves several different DNA sequence elements. Sequences within the upstream promoter region, the enhancer region in the $J_H-C\mu$ intron, and intragenic sequences are all involved in tissue-specific gene expression (20, 21). Activation of the IgH enhancer is mediated by *trans*-acting cellular factors (22–24). A number of different nuclear proteins that bind to distinct sites within the IgH enhancer have been identified (25). While *in vivo* experiments provide evidence for a factor binding to the Ig enhancer only in B-lineage cells, the enhancer-binding proteins identified *in vitro* are expressed in various cell types (26). DNA-binding proteins recognizing shared motifs in promoters or enhancers of specific sets of genes are the prime candidates for regulation of coordinate gene expression during differentiation. A *trans*-acting repressor competing for binding to the same sites or recognizing different shared sequences might account for the inhibition of expression of the same set of genes, as is observed in the anti- μ suppression model.

The qualitative and quantitative changes in Ig expression during B cell differentiation depend on posttranscriptional mechanisms as well as the rate of transcription. For example, the increased ratio of μ s to μ m mature mRNA that occurs in LPS-stimulated cells is controlled by a change in the preferred endonucleolytic cleavage site from 3' to 5' of the μ m exon (27). The importance of posttranscriptional regulation is also suggested by the observation that while the μ chain mRNA content of B lymphocytes and plasma cells differs as much as 100-fold, the nuclear transcription rate differs by only 2–5-fold (5). Chen-Bettecken et al. (8) observed the same kinds of differences in their comparisons of μ chain content and nuclear transcription rates of cells stimulated by LPS in the presence or absence of anti- μ , and suggested that posttranscriptional modification might be the major mechanism involved in anti- μ suppression. We believe that this is unlikely. First, the half-life of mature μ chain mRNA is long (~20 h [28]) in comparison to the time required for transcription (~30 nucleotides per second, or 20 min for a 30-kb unprocessed μ mRNA [29]). Thus a small change in the rate of transcription can easily account for a large change in mRNA content. Second, it is simply difficult to envision how posttranscriptional RNA processing could selectively regulate the entire set of different transcripts involved in this model without interfering with the products of housekeeping genes.

The microtrabecular network in cells is composed of microtubules, intermediate filaments, and microfilaments (30). In neonatal cells, antigenic suppression of differentiation is not affected by inhibitors of microtubules or microfilaments, but high-zone antigenic suppression of adult cells apparently requires both to be intact (18, 19). Our data indicate that suppression by anti- μ appears to require intact microtubules but not microfilaments. Desaynard (18) concluded that inhibition of high-zone antigen suppression by colchicine reflected a requirement for endocytosis in which microtubules were required. However, anti- μ antibodies coupled to beads are as effective as soluble antibody in stimulating B cells in the absence of LPS, indicating that endocytosis is not required for this activity (1). We demonstrate in this report that anti- μ antibodies coupled to beads are as effective as soluble anti- μ in suppressing differentiation in LPS-stimulated cul-

tures. Microtubules may be necessary for the functioning of a second messenger system which conveys the suppressive signal to the nucleus and are therefore required during the inductive phase of suppression.

Summary

Bivalent anti- μ antibodies suppress LPS-driven B cell differentiation by inhibiting the coordinate activation of a family of differentiation-related genes, including those encoding the heavy, light, and J chains of IgM. We have shown that the presence of inhibitors of RNA or protein synthesis during a pulse with anti- μ can interfere with induction of suppression. We suggest that suppression is mediated by a *trans*-acting repressor protein with specificity for common motifs in regulatory regions of each of these genes.

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