Effects of anti-IgM suppression on polyclonally activated murine B cells: analysis of immunoglobulin mRNA, gene specific nuclear factors and cell cycle distribution

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Received June 28, 1989; Revised and Accepted October 10, 1989

ABSTRACT

Polyclonal activation of murine B cells with bacterial lipopolysaccharide (LPS) and dextran sulfate (DxS) results in cell proliferation as well as increased immunoglobulin gene transcription and antibody secretion. When added to B cell cultures during mitogen activation, anti- μ antibody suppresses the rate of proliferation and immunoglobulin gene expression. Using this model system we show that co-cultures of B cells with LPS/DxS and anti- μ resulted in a decrease of both μ and κ chain mRNA. Suppression did not prevent B cell entry into cycle nor a significant alteration in the distribution of cells in phases of cell cycle, although it did prolong the cycle transit time in a dose dependent fashion as determined by bromodeoxyuridine pulse labelling. Analysis of B cell specific nuclear binding factors, which previously have been shown to be important in regulating immunoglobulin gene transcription were examined. Results show that the kappa-specific enhancer binding activity of NF- κ B was induced in activated as well as suppressed cultures. The lymphoid specific factor NF-A2, which recognizes the octamer sequence motif in the promoters of immunoglobulin genes, was induced by the polyclonal activation but was selectively lost in extracts from suppressed cells. Thus, specific regulation of the nuclear factor which plays a critical role in both heavy and light chain immunoglobulin gene expression may contribute to the transcriptional suppression observed in anti- μ treated B cells.

Introduction

Polyclonal activation of murine B cells with bacterial lipopolysaccharide (LPS) alone or in conjunction with dextran sulfate (DxS) results in cell proliferation and differentiation (1-3). The differentiation step involves increased immunoglobulin gene transcription and results in high rate immunoglobulin secretion. Mitogen induced differentiation can be suppressed by the addition of anti-immunoglobulin and has been used as a model to study down regulation of B cell differentiation (4-7). When anti- μ antibody was added at the initiation of culture along with LPS, or even up to 48 hours after culture initiation, B cell differentiation was shown to be inhibited. B cell proliferation was affected to a variable extent depending upon the use of intact or F(ab')₂ antibodies. Current evidence suggests that the inhibition of proliferation is mediated by the binding of

antibodies via the Fc receptor on B cells (8-15). It has been speculated that anti-immunoglobulin suppression may be representative of physiologic mechanisms wherein antigen activated B cells are prevented from terminal differentiation to plasma cells, return to quiescence, and subsequently turn into memory cells (7).

Anti- μ suppressed splenic B cell cultures have decreased steady-state levels of mRNA coding for κ and μ immunoglobulin molecules. It appears the anti- μ inhibition is selective for a restricted group of mRNA's, since the message levels coding for other proteins like actin and MHC class I and II molecules are unaffected (6,16). More recently, it has been demonstrated that the suppression directly affects transcriptional control of immunoglobulin genes (17).

Both heavy and light chain immunoglobulin genes contain regulatory elements responsible for modulating expression in a tissue-specific fashion: the 5' upstream promoter elements and the enhancer located within the JC intron (18-24). The same octanucleotide sequence motif is present in heavy and light chain promoter elements, as well as the heavy chain intron enhancer (19,25-28). This element is important in the tissue specific and developmental stage of immunoglobulin gene expression. Two nuclear factors, designated NF-A1 and NF-A2, have been shown to bind the octanucleotide sequence (29,30). Only NF-A2 shows lymphoid specificity, and has been shown to be an important mediator of tissue specific promoter activity of both heavy chain and light chain genes, as well as enhancer activity of the heavy chain gene. Within the κ enhancer, one sequence motif which appears essential for enhancer function is identical to the 11 bp SV-40 core enhancer sequence, and a specific factor whose binding correlates with κ expression has been identified and designated NF- κ B (31-33). This factor has been demonstrated in mature B cell extracts and shown to be inducible by LPS in the pre-B cell line, 70Z/3 as well as normal B lymphocytes (34). In this study we have examined the effects of anti- μ suppression on LPS/DxS activated splenic B cells as assessed by immunoglobulin mRNA production, entry into cell cycle, and cell cycle transit time. To gain some insight into the role of specific

nuclear factors when B cells are affected by regulatory signals which can specifically modulate immunoglobulin gene expression, the relative levels of nuclear factors obtained from activated and anti- μ suppressed splenic B cells were examined.

MATERIALS AND METHODS

Animals

Virus free BALB/c mice were obtained under NCI contract through the Veteran's Administration. Animals were used at 8 to 12 weeks of age and were monitored for Sendai and MHV titers prior use.

Preparation of cells and culture conditions

Single cell suspensions of spleens were made by gently pressing tissue fragments through 40 mesh screens. Cell preparations were prepared in RPMI 1640 supplemented with 10% fetal calf serum (RPMI-FCS). Red blood cells were lysed by incubation in HEPES buffered ammonium chloride. Following erythrocyte lysis, cells were washed twice with RPMI-FCS. After washing, spleen cell populations were enriched for B lymphocytes by panning on tissue culture flasks in Tris buffered hanks containing 3% BSA as previously described (35). Cell populations were greater than 95% surface immunoglobulin positive after this procedure as determined by flow cytometric analysis and less than 3% of the cells had exited G₀ as determined by acridine orange staining (36,37) and flow cytometric analysis. The splenic B cells were cultured in RPMI-1640 containing 10% FCS, 50 μ g/ml gentamycin, 5×10^{-5} M 2-ME, and 2mM L-glutamine at 1×10^6 cells/ml. Polyclonal activation of B cells utilized 50 μ g/ml LPS from E. coli (055:B5) and 20 μ g/ml dextran sulfate. Cells were co-cultured with affinity purified goat anti-mouse IgM (Southern Biotechnology, Birmingham, Al), as indicated. 3 H-TdR uptake was assessed after 72 hours of culture by pulsing 100 μ l cultures with 0.5 μ C 3 H-TdR (6.7 μ C/mM) for four hours and harvesting cultures with an automated harvester onto glass filter paper followed by liquid scintillation counting.

Flow cytometric analysis

For cell cycle analysis, cells were cultured with LPS/DxS plus or minus anti-IgM for 72 hours. Then, 1×10^6 cells were stained

with acridine orange as described Darzynkiewicz et al (36,37). Cells were analyzed using FACS model 440 (Becton-Dickensen, Sunnyvale, CA.) and Electric Disk Software (Wayne Moore, Stanford University) was used for dual parameter histogram analysis. Cells were gated by forward and 90 degree light scatter; 20,000 cells were analyzed for each population. For cell cycle kinetics, 48 hour cultures were incubated for 1 hour with $10\mu\text{M}$ BrdU (Sigma, St. Louis, Mo.) at 1×10^6 cells/ml, then stained immediately or recultured as before prior to staining at 6 hour intervals. Cells were stained with fluorescein conjugated B44 anti-BrdU monoclonal antibody following the protocol provided by Becton-Dickensen. Cells were counter stained with propidium iodide and analyzed using the FACS. Gated analysis of dual parameter histograms of red versus green fluorescence was performed as described by Sasaki, et al. (38). Cyclical curves of labelling index versus time were used to calculate approximate cell cycle time as described in the text.

Northern blot analysis.

Total RNA was prepared by cell lysis with 0.5% NP-40 in Tris, NaCl, EDTA buffer (10mM, 850mM, and 50mM respectively) followed by phenol:chloroform extraction and ethanol precipitation as previously described (39). Five micrograms of RNA were glyoxylated (40) and separated on 0.8% agarose using 10mM phosphate buffer. The gel was washed in 50mM NaOH containing $\mu\text{g/ml}$ ethidium bromide for 30 minutes followed by two 10-minute washes in 0.2M sodium acetate, transferred to Gene Screen Plus membranes by capillary transfer, allowed to air dry and then prehybridized with 50% formamide, 1% SDS, 1M NaCl, 10% dextran sulfate, and $100\mu\text{g/ml}$ denatured herring sperm DNA. Blots were probed with u specific (PMK1,41) and κ specific (pECK,42) nick translated cDNA probes overnight in the buffer described above. After washing the blot in 2X SSC-0.1% SDS, hybridization was detected by overnight exposure to XAR-5 film using intensifying screens at -70°C .

Nuclear Extracts and DNA--protein binding assays.

Nuclear extracts were prepared as described by Digman, et al (43). All buffers contained $100\mu\text{M}$ phenylmethylsulfonyl fluoride (PMSF), $0.5\mu\text{g/ml}$ leupeptin and $0.01\mu\text{M}$ pepstatin to inhibit

proteases. Protein concentrations were determined as described by Lowry (44). Nuclear binding factors were analyzed by their altered mobility on acrylamide gels as previously described (34). Double stranded oligonucleotides used included a 34 bp sequence containing the NF- κ B binding site (34, sense strand: 5'-TCTCAACAGAGGGGACTTCCGAGAGCCATCTGG-3') and a 27 bp duplex containing the octamer sequence motif in the context of the 70Z/3 kappa promoter sequence (25, sense strand: 5'-GTATCTTGC-GATTTGCATATTACATTT-3'). The NF-PB DNA fragment contains an AP-1 consensus sequence which is located 200 bp 5' of the octamer sequence in the kappa gene (A.M. and B.V.N., in preparation). Oligonucleotides were end labelled with 32 P-ATP and polynucleotide kinase.

RESULTS

Proliferation and Cell Cycle Analysis of Anti- μ Suppressed B Cell Cultures.

In order to assess the effect of anti- μ antibody on the proliferation of activated B cells, splenic B cells were enriched by panning, then cultured with LPS/DxS alone or with increasing concentrations of affinity purified goat anti- μ . Proliferation was assessed by tritiated thymidine uptake at 72 hours and confirmed by actual cell counts, listed as per cent

Table I

Anti-IgM Inhibition of Proliferation			
Treatment (+ μ g/ml anti-IgM)	Mean CPM+S.D. ^a	%Suppression ^b	% Recovery ^c
None	9561+1122	N.D.	N.D.
LPS/DxS	150767+5917	0.0	255
LPS/DxS+5 μ g	99453+4120	34.0	218
LPS/DxS+10 μ g	77762+8373	48.4	158
LPS/DxS+20 μ g	47088+2121	68.8	67.5
LPS/DxS+50 μ g	7430+1048	95.1	N.D.

^a Tritiated thymidine uptake after a four hour pulse.

^b calculated as $(1 - \text{LPS/DxS} + \text{anti-IgM cpm} - \text{LPS/DxS alone cpm}) \times 100$

^c Percentage recovery calculated as total number of cells obtained at the end of culture divided by the input number of cells at the initiation of culture.

Table II

Cell Cycle Analysis of Anti-IgM Treatment				
Treatment	Position in Cell Cycle % of Cells ^a			
	G ₀	G ₁	S	G ₂ +M
LPS/DxS	13.2	63.7	10.4	12.7
LPS/DxS + 5μg anti-IgM	3.0	68.0	13.2	15.8
LPS/DxS + 10μg anti-IgM	3.3	58.5	17.6	20.6
LPS/DxS + 20μg anti-IgM	6.3	61.5	15.3	16.9

^a Cells were stained with acridine orange and analyzed using two color fluorescence by flow cytometry.

recovery, from parallel bulk cultures. Data from a representative of over ten experiments are shown in Table I. Anti- μ treated co-cultured resulted in a dose dependent decrease in both proliferation and actual cell recovery.

Although thymidine incorporation is a useful measure of cell proliferation, it really reflects rates of de novo DNA synthesis, and does not address whether cells are capable of progressing through cell cycle. The cell cycle distribution of the LPS/DXS activated cultures with or without anti- μ was determined by staining the cells with acridine orange followed by flow cytometric cell cycle analysis. Monomeric acridine orange binds double-stranded native DNA and fluoresces green, whereas polymeric acridine orange binds single-stranded RNA and fluoresces red. Determination of red and green fluorescence in single cells by flow cytometric analysis permits categorization of cells into G₀, G₁, S, and G₂+M phases of cell cycle. As can be seen in Table II, contrary to the thymidine incorporation data, we did not observe a dose dependent decrease in the number of cells in cycle nor a retention of cells in any particular phase of cycle in any of three experiments. However, as seen in Table I, the actual number of cells recovered from bulk cultures of anti- μ treated cells was consistently lowered in a dose dependent manner. These results could be due to an increase in cell cycle transit time as the result of anti- μ treatment. Therefore, we decided to evaluate cell cycle kinetics by bromodeoxyuridine (BrdU) pulse labelling and staining of the DNA with the B44

monoclonal antibody directed against BrdU. B cells plus LPS/DXS alone or with either 5 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$ anti- μ were cultured for two days, washed once, incubated with 10 μM BrdU for one hour and then stained immediately or recultured with mitogen per the initial culture conditions for an additional 24-36 hours. Cells were stained with anti-BrdU, counter stained with propidium iodide to stain total DNA, and analyzed for two color fluorescence. BrdU pulsing results in the uptake of this thymidine analog by cells which are in S phase at the time of pulsing. The uptake can be directly determined based upon staining with the FITC conjugated monoclonal antibody against BrdU. A labelling index was calculated from dual parameter histograms based upon gated analysis. A gate was set around the cells which were positive for BrdU content but were also within the subpopulation of cells which contained 2X DNA. A second gate was set around all cells which contained 2X DNA and includes those cells positive and negative for BrdU green fluorescence staining. A labelling index was calculated by taking the number of cells positive for BrdU and 2X DNA and dividing by the number of all cells having 2X DNA. As the cells passed through G_2 and M, the green fluorescence positive cells are observed to progress back into G_1 with time, hence a decrease in the labelling index as the cells progressed through cycle. As the cells re-enter G_2 -M, the index again increases. Over time, graphic analysis of the labelling index generates a cyclical curve from which cell cycle transit time can be approximated (38). Cell cycle transit time correlates with the length of time for one complete cycle to be completed. The dual parameter histograms from the cells stained immediately following BrdU pulse are shown in Figure 1. Panel A is LPS/DxS alone and panels B and C are LPS/DxS with 5 and 10 $\mu\text{g/ml}$ anti-IgM, respectively. The numbers represent the percentages of cells positive for BrdU staining within the subpopulation containing 2X DNA (labelling index). It is readily apparent that the anti- μ co-culture resulted in a lower BrdU uptake in a dose dependent manner, i.e. anti-IgM resulted in a lower number of cells actively incorporating BrdU at the time of pulsing due to a slower cell cycle transit time. The approximate cell cycle time for these cells

was calculated to be 18 hours for LPS/DxS activated cells, 24 hours for LPS/DxS plus 5 $\mu\text{g/ml}$ anti- μ , and 32 hours for LPS/DxS plus 10 $\mu\text{g/ml}$ anti- μ . We conclude that while B cells can enter cycle in the presence of LPS/DxS plus anti- μ , the cell cycle transit time is increased.

Immunoglobulin mRNA Content of Anti- μ Suppressed B Cells.

Parallel bulk cultures to proliferation assays were established for preparation of mRNA to determine the steady state levels of immunoglobulin transcripts. Northern blot analysis of mRNA from 72 hour cultures demonstrates that the anti- μ treatment caused a dose dependent loss of both κ and μ mRNA (Figure 2). These results were consistent in over 5 experiments, and is in agreement with the suppressive effects on immunoglobulin gene expression seen by others (4-15). Thus, anti- μ treatment has a significant inhibitory effect on immunoglobulin expression, and was not due to cell death since the cells remain viable and in cycle; and as previously reported, without a marked effect on other gene expression (6,16).

Immunoglobulin-specific Nuclear Binding Factors in Anti- μ Suppressed B cells.

We have previously shown the induction of NF- κ B binding to the κ enhancer sequence in extracts derived from LPS/DxS activated normal splenic B cells (34). The nuclear factor which is specifically induced in lymphoid cells to bind the octamer sequence motif, NF-A2, is also inducible in splenic B cells (see Figure 3 and 28). Therefore, we wanted to evaluate if the induction of these factor binding activities was affected by anti- μ suppression of the B cell activation. Enriched splenic B cells were cultured with LPS/DxS alone or with 10 $\mu\text{g/ml}$ of goat anti- μ . Cells were harvested at 72 hours and used to prepare nuclear extracts. To check for the transcriptional effects described above, an aliquot was also used for mRNA analysis.

Figure 1. Cell cycle kinetics analysis. B cells were cultured with LPS/DxS alone (A), LPS/DxS + 5 $\mu\text{g/ml}$ anti- μ (B), or LPS/DxS + 10 $\mu\text{g/ml}$ anti- μ (C). On day two of culture cells were pulsed for one hour with 10 μM of BrdU and then stained immediately with the FITC conjugated monoclonal anti-BrdU and analyzed using the FACS (see methods). The number adjacent to the panel number represents the percentage of labelled mitoses.

Figure 2. Northern blot of total cellular RNA of LPS/DxS activated and anti- μ treated cells. Three days after initiation of culture, total RNA was prepared, 5 μ g RNA from each culture was glyoxalated (equal loading of RNA was confirmed by ethidium bromide staining) and separated by agarose electrophoresis, transferred to Gene Screen, and probed for μ and κ mRNA using nick translated cDNA probes. The upper band in the kappa RNA blot represents non-specific binding to the 28S rRNA band due to use of intact plasmid for probing.

Northern blot analysis showed a significant decrease in mRNA for both μ and κ in the anti- μ treated cells, as expected (not shown). Nuclear DNA binding factors were assayed by the electrophoretic mobility shift assay using end labelled double stranded oligonucleotides containing the recognition sequence of NF- κ B or NF-A2 (see Methods). Equal amounts of protein from each extract were assayed. We have confirmed the specificity of factor binding to oligonucleotides by both unlabelled competitor binding and footprinting experiments (data not shown). As seen in Figure 3, nuclear extracts from fresh resting B cells did not demonstrate any detectable binding with the NF- κ B specific oligonucleotide sequence; whereas LPS/DxS treated cells demonstrated significant binding activity. The lower shifted band is variably present in extracts of high protein concentration, and has been noted elsewhere (33,34). Nuclear extract from the

Figure 3. Nuclear factor content of extracts derived from fresh unactivated B cells (B cell⁻), LPS/DxS activated and LPS/DxS + anti- μ splenic B cells. The binding activities of NF-A1, NF-A2, NF- κ B and NF-PB from activated and suppressed extracts were determined by mobility shift assays (see Methods). Specific protein-DNA complexes are labelled.

anti- μ treated cells also contained significant binding activity, although we reproducibly noted a minor loss of activity. Thus, nuclear extracts from suppressed B cell cultures retain a significant NF- κ B binding activity.

The octanucleotide specific binding factors (NF-A1 and NF-A2) were determined using a double stranded oligonucleotide containing the octamer sequence motif within the context of the 70Z/3 cell line κ gene promoter. The results demonstrate little binding activity to the octamer sequence in resting, unstimulated B cells. In both stimulated and suppressed B cell cultures, the ubiquitous octanucleotide binding factor (NF-A1) was present at comparable levels. Little NF-A1 binding activity was observed in resting, unstimulated B cells. We cannot rule out possible degradation of extracts from these cells to account for

lack of NF-A1 binding activity. However, we favor an alternative explanation in that these cells are transcriptionally quiescent as assessed by acridine orange staining. Therefore, minimal amounts of NF-A1 would be detectable in comparison to transcriptionally active stimulated cells. However, a significant loss in the lymphoid-specific NF-A2 binding activity was consistently observed (three separate experiments) in the suppressed culture. Thus, the decreased binding activity observed for NF-A2 appears to be quite selective. The intermediate band has been observed previously (45) and does not appear to be affected by anti- μ co-culture. As another control, we examined the effects of the suppression on an additional factor binding activity we have characterized, designated NF-PB, as an AP-1 consensus sequence located 200 bp 5' of the κ octamer sequence (A.M. and B.V.M., in preparation). Suppression has no effect on the binding activity of this factor, thus providing an additional control for equal loading and integrity of the two extracts.

The absence of detectable NF-A2 binding activity in suppressed extracts could reflect either inhibition of the factor induction, specific proteolysis of NF-A2, the presence of an inhibitor molecule which displaces or prevents NF-A2 binding, or a specific covalent modification of the factor. To evaluate some of these possibilities, nuclear extracts from activated cells were mixed with extracts from anti- μ suppressed cells, incubated for 30 min, and then assayed by the mobility shift assay (Figure 4). Lane 1 represents the binding of LPS/DxS activated cell extracts to the octanucleotide sequence, and lane 2 the binding of anti- μ treated extracts. In lanes 3-6 binding reactions contained 10 μ g suppressed extract incubated with increasing amounts of activated extracts. There does not appear to be any effect of the extract from suppressed cells on the binding activity of extracts from the activated cells. Therefore, these in vitro results suggest the decreased binding of NF-A2 in suppressed extracts is not due to a proteolytic activity or an inhibitory factor, but rather is likely due to a lack of NF-A2 induction. That NF-A2 requires the induction of new protein synthesis in activated cells (as opposed to modification of a preexisting factor) is supported by the fact that the acti-

Figure 4. Effects of extracts from anti- μ suppressed B cells on NF-A2 binding activity in activated B cell extracts. Increasing amounts of nuclear extract from the LPS/DxS activated B cells were preincubated with anti- μ suppressed extracts, and examined for NF-A1 and NF-A2 binding by the mobility shift assay. Lane 1, LPS/DxS activated B cell extract; lane 2, LPS/DxS + anti- μ treated B cell extract; lanes 3-6, 10 μ g of anti-IgM treated extract and increasing amounts (2.5, 5.0, 7.5 μ g) of LPS/DxS activated B cell extract brought to 20 μ g total protein with BSA; lanes 7-10, same as lanes 3-6 but brought to 20 μ g total protein with extract from 70Z/3 noninduced cells.

vation of NF-A2 binding activity is inhibited by cyclohexamide (29 and A.M., unpublished)

DISCUSSION

When anti- μ antibody is included in the culture of LPS activated B lymphocytes, proliferation has been shown to be affected to a variable extent (4-15, and this study). The diversity of proliferative response to anti- μ antibody is not entirely understood; however, there seems to be a correlation between the proliferative effects and the source of antibody used. Results from several laboratories indicate that the reduced proliferation seen by anti- μ treatment of activated B cells is related to the non-variable, Fc portion of the antibody molecule (8-15).

Relevant to this conclusion is that the inclusion of only the F(ab')₂ portion of the antibody along with LPS in B cell cultures has little effect on proliferation (7,12). Thus, it is likely that the reduction in proliferation is mediated by the simultaneous binding of antibody molecules to the cell surface immunoglobulin and Fc receptors. We have not attempted to resolve this issue in the current study.

In order to determine if the suppression of immunoglobulin expression was germane to the maturation process, it was necessary to establish that the B cells were able to traverse the cell cycle and proliferate in the culture conditions used. Flow cytometric analysis of suppressed and activated B cell cultures indicated that both populations had a similar distribution for each phase of the cell cycle. This is important to eliminate the possibility that the anti- μ suppressive effect is the result of preventing a large percentage of cells from exiting G₀ and progressing through cycle. Also, the fact that these cells entered cycle precludes any toxic or lethal effects as the result of anti- μ co-culture. The BrdU pulse experiment demonstrates the capacity of suppressed cells to replicate; albeit with a longer transit time through the cycle. Since B cells stimulated by LPS can continue to differentiate, even when proliferation is not sustained at a high rate (39), it is likely that the anti- μ suppression mechanism is not the result of limiting rounds of replication.

Splenic B cells, as well as pre-B cell lines such as the 70Z/3 line, respond to activation with LPS treatment, inducing both NF- κ B binding activity and expression of κ (34). There is strong evidence that the activation of the κ enhancer is mediated by the induction of NF- κ B binding to the 11 bp core enhancer sequence (33,34). We have observed that significant NF- κ B binding activity can be detected even in the nuclear extracts from anti- μ suppressed B cells, which indicates that the signal responsible for κ gene activation has been delivered. We have also observed the specific inhibition of a transcription factor crucial for the expression of immunoglobulin genes, NF-A2. It is noteworthy that the only critical regulatory element shared by both the heavy and light chain immunoglobulin genes is the

octanucleotide located in the promoter of both, and the enhancer of the heavy chain. If, as our data suggests, NF-A2 activity is lost, the promoter activity will be depressed or even abrogated with the loss of a synergistic effect with the corresponding enhancer.

The absence of NF-A2 binding activity may reflect a transcriptional block in its expression since we and others (29) have observed that NF-A2 induction is cyclohexamide sensitive. This is in contrast with NF- κ B, whose binding activity is not cyclohexamide sensitive (33) but rather appears to require modification of a pre-existing inhibitory factor (I κ B) to activate its transport to the nucleus, and subsequent DNA binding (46). Because mixing suppressed and activated extract did not significantly diminish NF-A2 binding (Figure 4), it seems more likely that the anti- μ suppressive effect is blocking the induction of NF-A2 synthesis. An alternative explanation may be that there exists an inhibitory molecule for NF-A2 such as has been shown to regulate NF- κ B.

Flahart and Lawton (47) have demonstrated that the induction of suppression can be prevented by the inclusion of inhibitors of RNA and protein synthesis during the first 24 hours of culture with anti- μ . This implies the existence of an inducible suppressor, as the result of anti- μ co-culture, that could act on the regulatory DNA sequences of immunoglobulin genes or inhibit binding of proteins such as NF-A2. We have observed, as did Chen-Bettecken et al (7), that mRNA suppression was still evident when the anti- μ was added as late as 24 hours after the initiation of culture. This could be interpreted to support the observations of Flahart and Lawton (47) as to the induction of a suppressor molecule. However, when we mixed extracts from LPS/DxS alone cultures with those also receiving anti- μ , we could not detect any proteolytic effects on NF-A2 nor any inhibitory molecules which would displace or otherwise prevent NF-A2 binding. In any event, the data presented here place NF-A2 in a pivotal role in the regulation of transcription. Final interpretation of this data will require direct analysis of mRNA for NF-A2, and these studies are currently being undertaken.

A current hypothesis proposes that at some stage in B cell

development, immunoglobulin enhancers become dispensable. Atchison and Perry (48) suggested that developmental changes in chromatin structure and/or methylation of immunoglobulin loci may be ultimately responsible for rendering the enhancer dispensable. It is tempting to speculate that the NF-A2 induction may be essential to establish and maintain transcription in mature B cells. Thus NF-A2 binding represents a central controlling element which can exert its effects on both heavy and light chain genes, which share the need for its binding to maintain maximum expression. Results presented here would suggest it plays an important role in immunoglobulin gene expression associated with B cell differentiation. Our results also demonstrate that while NF- κ B binding activity may be necessary for gene expression at early stages of differentiation, it is by itself not sufficient to maintain high levels of immunoglobulin gene expression.

The data presented here can explain the loss of immunoglobulin specific mRNA expression in anti- μ suppressed B cells. The suppression is specific for immunoglobulin gene loci since neither class I and class II MHC genes nor actin gene mRNAs are suppressed (6,16). The point of regulation would appear to be at the transcriptional level since in nuclear run-on assays performed by Yuan (17), the transcriptional rate of the μ gene returned to basal levels in anti- μ treated cells. In a similar model of anti- μ suppression, Chen-Bettecken, et al (7) used an F(ab')₂ anti- μ antibody and concluded that early loss of secreted μ levels was a post-transcriptional event. Our data does not rule out this possibility, but suggests there may be additional factors involved in the μ chain transcriptional regulation. Alternatively, the use of intact anti- μ may deliver either a stronger suppressive signal or additional signals mediated by Fc receptor binding. A direct comparison of these two systems may be required to evaluate the importance of these two regulatory events.

ACKNOWLEDGMENTS

This work was supported by funds from the Medical Research Service, Department of Veterans Affairs (D.L.) and by grant GM 37687 from the National Institutes of Health (B.V.N.)

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REFERENCES

1. Anderson, J., Sjöberg, O. and Møller, G. (1972) *Transplant. Rev.* **11**, 131-177.
2. Kettman, J. and Wetzel, M. (1980) *Immunol. Meth.* **39**, 203-222.
3. Yuan, D. and Tucker, P. W. (1984) *J. Exp. Med.* **160**, 564-583.
4. Anderson, J., Coutinho, A. and Melchers, F. (1978) *Eur. J. Immunol.* **8**, 336-343.
5. Kearny, J.F., Klein, J., Beckman, D.E., Cooper M.D. and Lawton, A.R. (1978) *J. Immunol.* **120**, 158-166.
6. Leanderson, T., and Hsu, E. (1985) *Eur. J. Immunol.* **15**, 641-643.
7. Chen-Bettecken, U., Wecker, E. and Schimpl, A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7384-7388.
8. Sidman, C.L., and Unanue, E.R. (1986) *J. Exp. Med.* **144**, 882-896.
9. Parker, D. (1975) *Nature* **258**, 361-363.
10. Sidman, C.L. and Unanue, E.R. (1979) *J. Immunol.* **122**, 406-413.
11. Tony, H.P. and Schimpl, A. (1980) *Eur. J. Immunol.* **10**, 726-729.
12. Parker, D.C. (1980) *Immunol. Rev.* **52**, 115-139.
13. Seickmann, D.G. (1980) *Immunol. Rev.* **52**, 181-210.
14. Vitetta, E.S., Pure, P., Isakson, L. and Uhr, J.W. (1980) *Immunol. Rev.* **52**, 211-231.
15. Phillips, N.E. and Parker, D.C. (1983) *J. Immunol.* **130**, 602-606.
16. Flahart, R.E. and Lawton, A.R. (1987) *J. Mol. Cell. Immunol.* **3**, 61-68.
17. Yuan, D. (1987) *J. Mol. Cell. Immunol.* **3**, 133-144.
18. Gillies, S.D., Morrison, S.L. V.T.O. and Tonegawa, S. (1983) *Cell* **33**, 717-728.
19. Falkner, F.G. and Zachau, H.G. (1984) *Nature* **310**, 71-74.
20. Bergman, F.D., Rice, D., Grosschedl, R. and Baltimore D. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7041-7045.
21. Queen, C. and Baltimore, D. (1983) *Cell* **33**, 741-748.
22. Queen, C. and Stafford, J. (1984) *Mol. Cell. Biol.* **4**, 1042-1049.
23. Picard, D. and Schaffner, W. (1984) *Nature* **307**, 80-82.
24. Picard, D. and Schaffner, W. (1985) *EMBO* **4**, 2831-2838.
25. Parslow, T.G., Blair, D.L., Murphy, W.J. and Granner, D.K. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2650-2654.
26. Singh, H., Sen, R., Baltimore, D. and Sharp, P.A. (1986) *Nature* **319**, 154-158.
27. Mason, J.O., Williams, G.T. and Neuberger, M.S. (1985) *Cell* **41**, 479-487.
28. Landolfi, N.F., Capra, J.D. and Tucker, P.W. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3851-3855.
29. Staudt, L.M., Singh, H., Sen, R., Wirth, T., Sharp, P.A. and Baltimore, D. (1986) *Nature* **323**, 640-643.
30. Scheidereit, C., Heguy, A. and Roeder, R.G. (1987) *Cell* **51**, 783-793.
31. Hromas, R. and Van Ness, B. (1986) *Nuc. Acids Res.* **14**, 4837-4848.
32. Sen, R. and Baltimore, D. (1986) *Cell* **46**, 705-716.
33. Sen, R. and Baltimore, D. (1986) *Cell* **47**, 921-928.
34. Hromas, R., Pauli, U., Marcuzzi, A., Lafrenz, D., Nick, H., Stein, J., Stein, G. and Van Ness, B. (1988) *Nuc. Acids Res.* **16**, 953-967.
35. Severson, C. D., Burg, D. L., Lafrenz, D. and Feldbush, T.L. (1987) *Immunol. Lett.* **15**, 291-295.
36. Darzynkiewicz, Z., Sharpless, T., Stalano-Coico, L., and Melamed, M.R. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6696-6699.
37. Darzynkiewicz, Z., Traganos, F., Sharpless, T. and Melamed, M.R. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2881-2884.
38. Sasaki, K., Murakami, T., Ogino, T., Takahashi, M. and Kawasaki, S. (1986) *Cytometry* **7**, 391-395.
39. Lafrenz, D., Koretz, S., Stratte, P.T., Ward, R.B. and Strober, S. (1982) *J. Immunol.* **129**, 1329-1335.
40. McMaster, G.K. and Carmichael, G.G. (1977) *Proc. Natl.*

- Acad. Sci. USA 74, 4835-4838.
41. Knapp, M.R., Liu, C., Newal, N., Ward, R.B., Tucker, P.W., Strober, S. and Blattner, F. (1982) Proc. Natl. Acad. Sci. USA 79, 2996-3000.
 42. Van Ness, B., Colecough, C., Perry, R.D. and Weigert, M. (1982) Proc. Natl. Acad. Sci. USA 79, 262-266.
 43. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nuc. Acids. Res. 11, 1475-1489.
 44. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
 45. Wirth, T., Staudt, L. and Baltimore, D. (1987) Nature 329, 174-178.
 46. Baeuerle, P.A. and Baltimore D. (1988) Science 242, 540-545.
 47. Flahart, R.E. and Lawton, A.R. (1987) J. Exp. Med. 166, 864-873.
 48. Atchison, M.L. and Perry, R.P. (1987) Cell 48, 121-128.