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Immunology

Characterization of a presecretory phase in B-cell differentiation

(B lymphocyte/lymphocyte activation/gene expression/mouse mammary tumor virus *env*/J chain)

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ABSTRACT **We have identified and characterized an inducible *in vitro* subclone of the CH12 B-cell lymphoma, CH12-LBK, which appears to represent a transitional phase in the B-cell differentiation pathway. This phase, which we call the "presecretory" phase, falls between replicating B cells that are not secreting antibodies and B cells that secrete antibody at a high rate. Presecretory cells are characterized by abundant steady-state levels of immunoglobulin and joining (J) chain transcripts and of protein but low levels of mouse mammary tumor virus envelope transcripts and low rates of immunoglobulin secretion. Additional stimulation is required for presecretory cells to differentiate into cells that secrete antibodies at a high rate. The existence of cells with this phenotype suggests that high-level expression of immunoglobulin and J-chain protein does not necessarily commit a B cell to polymerize and secrete multimeric immunoglobulin. Rather, other gene products, expressed after immunoglobulin and J-chain transcripts have been upregulated late in B-cell differentiation, appear responsible for inducing high rates of antibody secretion.**

The B-cell differentiation pathway has been divided into phases based on cell-cycle stage, presence of various cell-surface markers, and by the expression of immunoglobulin molecules (1–9). The expression of immunoglobulin molecules has been particularly well characterized throughout the course of B-cell differentiation (2, 4–6). Resting B cells transcribe the membrane form of IgM μ heavy-chain mRNA (μ_m) but do not contain detectable levels of the secreted form of IgM μ heavy-chain mRNA (μ_s). Upon stimulation *de novo* transcription of μ_m and μ_s is initiated, and these transcripts reach approximately equimolar levels in proliferating nonsecreting B cells. Subsequently there is a dramatic increase in the level of μ_s relative to μ_m. Increases in mRNA levels of the joining (J) chain, a molecule associated with the polymerization and secretion of multimeric immunoglobulin in differentiated B cells, are also observed. Immunoglobulin and J-chain protein levels increase in a similar fashion, and only after increases in J-chain protein occur can secreted immunoglobulin be detected (4). However, the lack of suitable B-cell tumor models and the asynchronous differentiation of mitogen-driven normal B cells (10) make it difficult to assess whether high rate antibody secretion directly results from increased levels of immunoglobulin and J-chain proteins or whether additional events are necessary for the initiation of immunoglobulin secretion.

We have characterized a B-cell lymphoma, CH12-LBK, which expresses abundant immunoglobulin and J-chain mRNA and protein but is not actively secreting antibody at a high rate. Stimulation with lipopolysaccharide (LPS) causes the cells to differentiate into cells that secrete antibody at a high rate. Differentiation is accompanied by a significant increase in the level of mouse mammary tumor proviral envelope (MMTV *env*) transcripts, an event associated with B-cell differentiation (11), but only modest increases in the levels of immunoglobulin and J-chain transcripts are seen. These results indicate that the secretory status of a B cell cannot be predicted on the expression of immunoglobulin and J-chain mRNA and protein. Indeed, the expression of J-chain protein does not appear to be the rate-limiting step in the differentiation of B cells to cells that secrete antibody at a high rate. Instead, other molecular events, induced upon LPS stimulation, are necessary for the initiation of immunoglobulin secretion in differentiating B cells.

MATERIALS AND METHODS

Culture and Stimulation of the CH12-LBK Line. The CH12 B-cell lymphoma was passaged as an *in vivo* ascites tumor as described (8). An *in vitro* clone, CH12-LBK, was isolated by culturing the *in vivo* line in Dulbecco's modified Eagle's medium supplemented as described and containing 10% fetal bovine serum (DMEM/FBS) (8, 12). DMEM/FBS was prepared using endotoxin free water from an Ultra 70 system (Zenon Environmental, Burlington, ON). The cultures were originally supplemented with thymocyte feeder cells and 25–50% conditioned medium from the *in vivo* CH12 line. An inducible subclone, designated CH12-LBK, was recloned and maintained in DMEM/FBS with 5×10^{-7} M 2-mercaptoethanol. CH12 and CH12-LBK cells were stimulated with LPS as described (11). Antibody-secreting cells were detected using a plaque forming cell (pfc) assay (8). IgM secretion was quantitated by ELISA as described (13).

RNA Transfer Blot Analysis. RNA transfer blots were performed as described (11). Probes used were pμ107 (C_μ) (14), pκ(11)[24] (C_κ) (15), pHF-1 (actin) (16), pSC34 (MMTV *env*) (17), and pAJ5 (J chain). The PAJ5 cDNA clone was isolated by screening a CH12 λgt10 library with a J-chain probe, pUCJc59 (18). The 800-base-pair cDNA clone, pAJ5, has a 5′ limit at the codon for amino acid 27 of the J-chain protein and extends into the 3′-untranslated region.

Metabolic Labeling and Immunoprecipitations. Cells (5×10^6) were labeled for 4 hr with 360 μCi (1 Ci = 37 GBq) of Tran^{35}S-label (ICN). Cell lysates and supernatants were immunoprecipitated with normal rabbit serum, rabbit anti-mouse IgM, or an affinity-purified rabbit anti-J chain antiserum [from T. Mosmann (DNAX, Palo Alto, CA)], described in detail (19). Where indicated, samples were reduced with 20 mM dithiothreitol for 30 min and alkylated with 60 mM iodoacetamide for 1 hr before precipitation (19). Antigen–antibody complexes were precipitated with protein A–Sepharose, and pellets were washed extensively with 0.1 M Tris, pH 7.4/0.15 M NaCl/0.05% Triton X-100 (20).

Alkaline Urea Gel Electrophoresis. Immunoprecipitates were solubilized in 9.0 M urea/0.025% bromophenol blue/1%

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Abbreviations: μ_s, secretory form of IgM μ heavy-chain; μ_m, membrane form of IgM μ heavy-chain; LPS, lipopolysaccharide; DMEM/FBS, Dulbecco's modified Eagle's medium/10% fetal bovine serum; pfc, plaque forming cells; MMTV *env*, mouse mammary tumor proviral envelope gene.

2-mercaptoethanol for 30 min at 37°C and were electrophoresed on reducing 8% polyacrylamide alkaline urea gels (21). Gels were soaked in Enlightning (Dupont/NEN), dried, and exposed to film.

RESULTS

CH12-LBK Cells Differentiate in Response to LPS. The frequency of pfc in LPS-stimulated CH12-LBK cells increases significantly with time over that of unstimulated cells (Fig. 1). This difference cannot be accounted for by preferential proliferation of IgM-secreting cells because the proliferative rate of unstimulated and LPS-stimulated cells is similar (Fig. 1; see also ref. 11). The maximum number of pfc attained in stimulated CH12 cells is similar to the number of pfc detected in a (CH12 × NS/0) hybridoma, 1C9. Because all hybrid cells are probably secreting immunoglobulin, these data suggest that the number of pfc seen in LPS-stimulated CH12 cells reflects the limitations of the pfc assay and not the maximum frequency of pfc in the population.

We next measured the rate of IgM secretion by unstimulated and LPS-stimulated CH12-LBK cells (Fig. 2). Unstimulated cells secrete small amounts of IgM. Whether all secreted immunoglobulin results from high rates of secretion by the small number of pfc or whether many cells secrete at low basal levels is not known. CH12-LBK cells stimulated for 48 hr with LPS secreted IgM at the highest rate, even though the frequency of pfc was similar to that seen at 24 hr (Fig. 2). Interestingly, while the secretory rate of the cells stimulated for 48 hr remained constant, the secretory rate of the cells stimulated for 24 hr increased over time. These results suggest that LPS stimulation induces a commitment to IgM

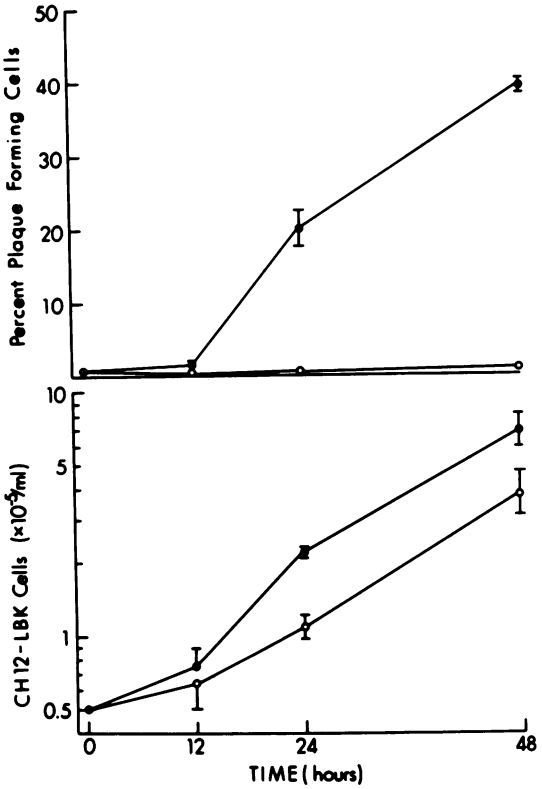


FIG. 1. Differentiation and proliferation of CH12-LBK cells cultured with (●) or without (○) LPS (50 μg/ml). (*Upper*) Differentiation of CH12-LBK cells, represented as the percentage of viable cells recovered from cultured cells that secrete antibody in a pfc assay. (*Lower*) Proliferation of CH12-LBK cells, represented as the number of viable cells recovered from cultures at the times indicated. Data are presented as mean ± SD of replicate cultures.

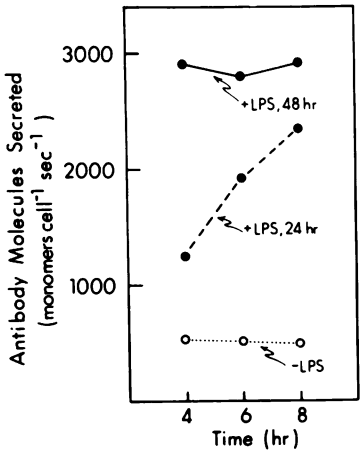
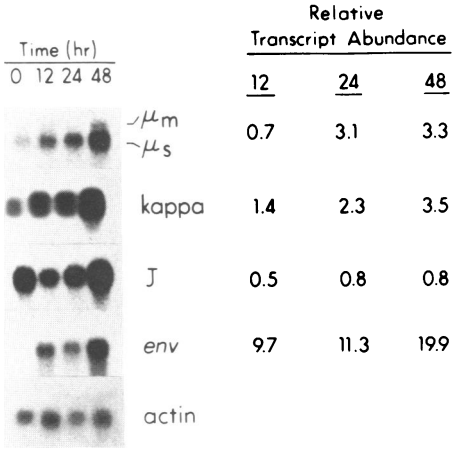


FIG. 2. Rate of secretion of IgM by LPS-stimulated and unstimulated CH12-LBK cells. Cells were stimulated for either 0, 24, or 48 hr with LPS and harvested, and 2×10^5 cells were placed in 1-ml cultures without LPS. Supernatants were collected at the indicated times, and the IgM was measured by ELISA. Secretion rate of monomeric IgM was calculated per cell·sec^{-1}, assuming an average molecular mass of 180 kDa per monomeric IgM. The number of immunoglobulin monomers secreted is expressed as an average secretory rate over time after reculture. No attempt was made to correct for the number of pfc in culture. Percentages of pfc of viable recovered cells were as follows: 0 hr, 3.2 ± 1.0%; 24 hr (+LPS), 42.3 ± 8.9%; 48 hr (+LPS), 37.7 ± 2.2%. Maximum rate of immunoglobulin secretion was 3200 IgM monomers cell^{-1}·sec^{-1}.

secretion within 24 hr and that the continued presence of LPS is not required for the cells to achieve their maximal secretory rates. We confirmed that the majority of secreted immunoglobulin from both unstimulated and stimulated cells is of high molecular weight and contains covalently associated J chain (data not shown).

RNA Transfer Blot Analysis of LPS-Induced CH12-LBK Differentiation. We next analyzed levels of immunoglobulin and J-chain transcripts in CH12-LBK cells stimulated with LPS for various times (Fig. 3). Although the frequency of



	Relative Transcript Abundance		
	12	24	48
μm, μs	0.7	3.1	3.3
kappa	1.4	2.3	3.5
J	0.5	0.8	0.8
env	9.7	11.3	19.9
actin			

FIG. 3. RNA transfer blot analysis of the steady-state levels of various mRNA transcripts in CH12-LBK cells during LPS-induced differentiation. CH12-LBK cells were stimulated with LPS for the indicated times and then collected. RNA was extracted and subjected to oligo(dT)-cellulose chromatography, and 3 μg of poly(A)$^+$ mRNA were resolved on a 1.6% agarose–formaldehyde gel. The RNA was transferred onto Nitroplus 2000 (Micron Separations, Fisher) and was probed with random-primed cDNA inserts of the plasmid probes for C_μ, C_κ, J chain, MMTV *env*, and actin. The percentages of pfc were: 0 hr, 7.0 ± 1.4%; 12 hr (+LPS), 3.7 ± 2.5%; 24 hr (+LPS), 54.4 ± 9.5%; 48 hr (+LPS), 28.6 ± 1.5%. The relative transcript abundance, as determined by densitometric scanning and normalized to actin expression, is listed in the right panel.

cells secreting antibody increased to 54% within 24 hr (Fig. 3), the corresponding increases in immunoglobulin and J-chain transcript levels, which normally accompany B-cell differentiation (4–6) were not seen. Immunoglobulin and J-chain transcripts were abundant in unstimulated cells, and the level of these transcripts increased only modestly in LPS-stimulated cells. These changes were measured by densitometer scanning and normalized with respect to actin expression and are shown in Fig. 3. The largest increases were seen 48 hr after LPS stimulation after maximal numbers of pfc were detected, but at a time when secretory rates are highest (see Fig. 2).

Although the abundance of immunoglobulin and J-chain transcripts did not change dramatically in response to LPS, the CH12-LBK line is not inert to the effects of LPS-induced transcriptional regulatory events. MMTV *env* transcripts, which are induced after stimulation of both CH12 cells and normal B cells with LPS (11), increased rapidly in abundance in the CH12-LBK line after LPS stimulation (Fig. 3).

CH12 and CH12-LBK Represent Distinct Phases in B-Cell Differentiation. We have previously shown that steady-state levels of μ_m and μ_s transcripts are represented in approximately equimolar concentrations in unstimulated *in vivo* CH12 cells, a characteristic shared by replicating nonsecreting B cells and that μ_s transcripts are upregulated dramatically during LPS-induced differentiation (17). We next compared immunoglobulin, J-chain, and MMTV *env* transcripts in unstimulated and LPS-stimulated *in vivo* CH12 and *in vitro* CH12-LBK lines. Two differences between the *in vivo* and *in vitro* CH12 lines were immediately apparent (Fig. 4). Steady-state levels of immunoglobulin and J chain were significantly more abundant in the CH12-LBK line, indicating that these transcripts were already upregulated. Moreover, immunoglobulin and J-chain transcripts increased more dramatically upon LPS stimulation in the CH12 line than in the CH12-LBK line, even though the populations contained similar numbers of pfc after LPS stimulation. These results suggest that the CH12 and CH12-LBK cell lines may be at different phases in the B-cell differentiation pathway.

Steady-state levels of immunoglobulin and J-chain transcripts were next analyzed in CH12 cells cultured in the absence or presence of LPS for 48 hr. CH12 cells did not differentiate into antibody-secreting cells in the absence of LPS, but levels of immunoglobulin and J-chain transcripts did increase (Fig. 5). Densitometric analysis revealed that at 48 hr, the ratio of relative transcript abundance in unstimulated

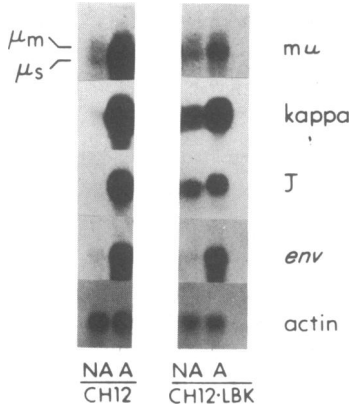


FIG. 4. Comparison of levels of various RNA transcripts in unstimulated (NA) and 48-hr LPS stimulated (A) *in vivo* CH12 cells (the parental cell line) and in the *in vitro* CH12-LBK line. Poly(A)$^+$ RNA (3 μg) was resolved on a 1.6% agarose–formaldehyde gel. RNA transfer blots were probed as described for Fig. 3. Frequencies of pfc were 1.6% and 47% for the unstimulated and stimulated CH12 cells, respectively, and 3.6% and 38.6% for CH12-LBK, respectively.

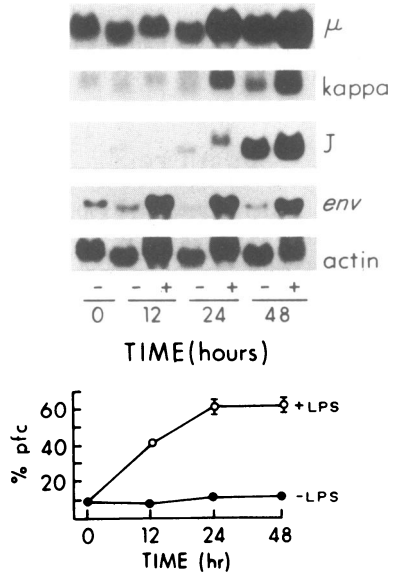


FIG. 5. Acquisition of a CH12-LBK-like phenotype by CH12 cells cultured in the absence of LPS. CH12 cells were cultured without or with LPS. At the indicated times, cells were harvested, and the number of pfc was counted; RNA was extracted and electrophoresed (25 μg per lane) on 1% agarose–formaldehyde gels, and RNA transcripts were analyzed using the indicated probes. Although the number of pfc did not increase in cells cultured without LPS, increases in μ and J-chain transcripts were seen. At 48 hr μ RNA had increased 2.1-fold vs. 7.3-fold in cultures without and with LPS, respectively (compared with 0 hr and normalized to actin controls). Increases in J-chain transcripts were 9.7-fold without LPS and 12.4-fold with LPS.

CH12 cells vs. LPS-stimulated CH12 cells approximated that seen in CH12-LBK cells for both immunoglobulin and J chain (Fig. 5). The steady-state levels of MMTV *env* transcripts did not, however, increase in the absence of LPS. These data indicate that the regulatory mechanisms controlling immunoglobulin and J-chain expression are distinct from those controlling MMTV *env* expression and that upregulation of MMTV *env* transcripts occurs after an initial upregulation of immunoglobulin and J-chain transcript levels.

Levels of Immunoglobulin and J-Chain Proteins in the CH12-LBK Line. Because immunoglobulin and J-chain mRNA were found in high abundance in CH12-LBK cells, we next determined whether the protein products were also expressed. Analyses were carried out on reducing alkaline urea gels to facilitate the identification of J chain in immunoprecipitates; J-chain protein migrates with a characteristic high mobility on these gels because of its negative charge and therefore can easily be resolved from κ light chain (22). Anti-J-chain antibody, which recognizes free but not immunoglobulin-associated J chain (19), precipitated J chain in the reduced lysates of both the unstimulated and stimulated cells (Fig. 6), indicating that J-chain protein is synthesized in both populations. In the unstimulated cell lysate, reduction and alkylation before precipitation with the anti-J-chain antisera resulted in more J chain in the immunoprecipitate. Reduction and alkylation may increase binding of anti-J-chain antibody to J chain, even in situations where J chain is not complexed with immunoglobulin (23). Indeed, because little J chain was found in anti-J chain precipitates or in anti-immunoglobulin precipitates from nonreduced cell lysates, J chain apparently could be associated with nonimmunoglobulin molecules in the unstimulated CH12-LBK cells. In contrast, in LPS-stimulated cell lysates, anti-J-chain antisera precipitated approximately equivalent amounts of J chain in both nonreduced and reduced and alkylated samples. Apparently, a pool of free J chain exists in the LPS-stimulated cells, which is not

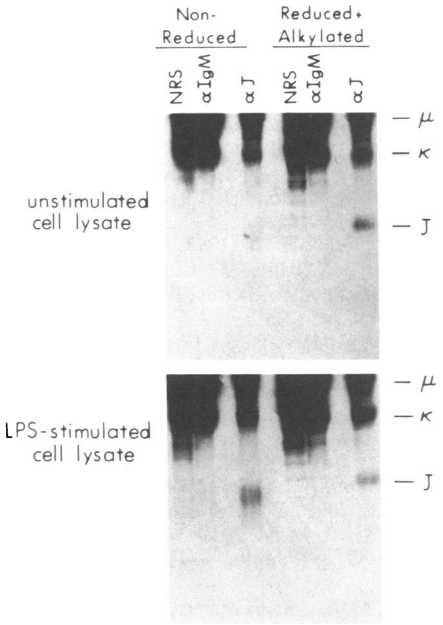


FIG. 6. Presence of J-chain protein in immunoprecipitates from both unstimulated and stimulated CH12-LBK cells. Unstimulated or LPS-stimulated (48 hr) CH12-LBK cells (5×10^6) were cultured for 1 hr in cysteine-free medium and then labeled with 360 μCi Tran^{35}S-label for 4 hr. Cells were lysed, and proteins were either directly immunoprecipitated (nonreduced) or were reduced and alkylated before immunoprecipitation. Precipitations were done using normal rabbit serum (NRS), rabbit anti-mouse IgM antisera (αIgM), and rabbit anti-J chain antisera (αJ) as described. Immunoprecipitates were solubilized and resolved on a reducing 8% alkaline urea gel.

present in the unstimulated CH12-LBK cells. Moreover, because anti-immunoglobulin immunoprecipitates from the nonreduced stimulated cell lysates did not contain much J chain, the results suggest that polymerization of J chain is a relatively late event in immunoglobulin secretion and that pentameric IgM molecules are secreted rapidly from these cells after assembly.

DISCUSSION

Upon stimulation, resting B cells enter the cell cycle and progress from replicating antibody-nonsecreting cells into cells that secrete antibodies at a high rate. Molecular analysis of an *in vitro* subclone of the CH12 lymphoma, CH12-LBK, suggests the existence of an additional transition phase, the presecretory phase, during the late stages of B-cell differentiation. This phase falls between the replicating B-cell phase, characterized by cells containing low, equimolar levels of μ_m and μ_s and little J-chain mRNA, and the antibody-secreting phase, in which B cells contain abundant μ_s, light-chain, and J-chain mRNA (4–6). Unstimulated CH12-LBK cells contain relatively high steady-state levels of immunoglobulin and J-chain mRNA, but they do not secrete antibody at a high rate until stimulated by LPS. Stimulated cells may express two to three times more immunoglobulin but rarely more J-chain transcripts than do the unstimulated cells. In contrast, transcripts of the MMTV *env* gene are dramatically upregulated (20-fold increase) after LPS stimulation, an event also observed in LPS-induced normal B-cell differentiation (11). From our data, MMTV *env* expression appears to be associated with the late stages of differentiation. Thus, in addition to inducing high-rate multimeric antibody secretion, LPS stimulation alters the program of genes expressed in CH12-LBK cells, providing more evidence that LPS induces a differentiative event in the CH12-LBK cell line.

The parental *in vivo* CH12 lymphoma shares the characteristics of replicating, antibody-nonsecreting cells (8, 17). Upon culturing in the absence of LPS, CH12 cells increase their steady-state levels of μ, κ, and J-chain transcripts. It is not clear what stimulus induces the expression of immunoglobulin and J-chain transcripts in the *in vivo* CH12 cells, but great care is taken to avoid endotoxin contamination. The expression of J chain in BCL_1 cells is induced by interleukin 2 (24), and J-chain expression may be stimulated in CH12 cells by an as-yet-unidentified autocrine factor produced by these cells. The acquisition of a CH12-LBK-like phenotype by CH12 cells supports the hypothesis that the presecretory phase is a true phase in B-cell differentiation. This phase would be obscured in most systems where B-cell differentiation was being analyzed, due to the asynchronous progression of B cells to antibody-secreting cells. Presecretory B cells, like antibody-secreting cells, have upregulated steady-state levels of immunoglobulin and J-chain mRNA and protein. Although the levels of immunoglobulin transcripts may be somewhat lower in presecretory cells than in antibody-secreting cells, these differences would not be apparent in populations that contained both cell types.

Abundant expression of immunoglobulin and J chain is typically associated with the increased secretory capacity of B cells during late stages of differentiation (for review, see ref. 25), and the process of polymerization and secretion of multimeric immunoglobulin in B lymphocytes have been hypothesized to depend on the presence of J chain (24, 25). The abundance of immunoglobulin and J chain in the unstimulated CH12-LBK line argues that although immunoglobulin secretion may depend on J-chain expression in differentiating B cells, the presence of J-chain protein alone does not predict the secretory status of a B cell. The expression of J chain in B cells not actively secreting polymeric immunoglobulin is not entirely without precedent. Low levels of J-chain transcript or protein have been detected in human pre-B cell lymphomas (23, 26, 27) and in unstimulated human peripheral B cells (23). In these studies, lack of expression of sufficient levels of immunoglobulin molecules in the cell at the time of J-chain expression likely prohibits antibody secretion. Unstimulated CH12-LBK cells, however, contain both immunoglobulin and J-chain molecules but still do not secrete antibody unless stimulated. This finding indicates that the expression of J chain cannot be the rate-limiting step for the induction of high rates of immunoglobulin secretion in B cells.

Although the presence of J chain may be important for polymerization and secretion of multimeric immunoglobulin, the fate and location of J chain within the cell may better indicate the secretory status of a B cells. Hadju *et al.* (23) found that J chain and immunoglobulin proteins may be localized in different compartments within the cell. In mitogen-stimulated human peripheral B cells, J chain is expressed first on free ribosomes in the cytoplasm and is only detected on the rough endoplasmic reticulum with μ chains at a time when the cells have begun to secrete immunoglobulin at high rates. The expression of J chain on free ribosomes would most likely not influence the secretory capacity of a B cell.

Unstimulated CH12-LBK cells express abundant amounts of J-chain protein. However, J chain appears to be associated with another molecule because reduction and alkylation of the cell lysate yields a greater amount of free J chain that can be precipitated by the anti-J-chain antiserum. Similar results were reported by Hadju *et al.* (23) with peripheral blood B cells. In CH12-LBK cells, anti-immunoglobulin precipitates from nonreduced cell lysates did not contain J chain, indicating that J is not complexed with IgM polymers. Therefore, in nonsecreting B cells, J chain could be synthesized but be complexed with a nonimmunoglobulin molecule that targets the J chain for degradation rather than for assembly and

secretion. Mosmann *et al.* (19, 22) reported that abundant levels of J-chain protein were synthesized in an IgA secretory mutant but that J chain was degraded by the cell at a rate comparable to the rate of secretion of J chain by normal IgA-secreting cells. Thus, there may be a degradative pathway for newly synthesized J chain that is not complexed with immunoglobulin, similar to the pre-Golgi proteolytic pathway recently described for unassociated subunits of the T-cell receptor (28).

Anti-J-chain antiserum immunoprecipitated about equal amounts of J chain from LPS-stimulated CH12-LBK cell lysates whether or not lysates were reduced and alkylated before precipitation. Thus, in immunoglobulin-secreting cells, J chain appears to be present primarily as a free protein, presumably ready to be complexed with immunoglobulin. However, little J chain is precipitated by anti-immunoglobulin from nonreduced cell lysates, suggesting that the polymerization of pentameric IgM is a relatively late event in the secretory pathway, an observation consistent with the results of Parkhouse and Askonas (29). LPS stimulation thus appears to induce a series of events that allow for the formation of IgM pentamers within the cells. Polymerizing enzymes, such as those suggested by Roth and Koshland (30) or proteins involved in the stabilization or localization of secretory IgM monomers and J chain could be induced during the late stages of B-cell differentiation. Presumably, the expression of such regulatory gene products, and not of J chain itself, is the rate-limiting step that determines the secretory capacity of differentiating B cells.

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