J CHAIN BIOSYNTHESIS IN PRE-B CELLS AND OTHER POSSIBLE PRECURSOR B CELLS*

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J chain was originally described as a highly acidic component of the polymeric secretory immunoglobulins IgM and IgA. Structural work has demonstrated a disulfide bridge between it and a cysteine present on the C terminus of both secretory μ and α heavy chains, and studies on the biosynthesis of these proteins have led to the view that J chain is, via this linkage, involved in the polymerization of multimeric immunoglobulins (1, 2).

A number of observations, however, would require certain modifications of this hypothesis. Some secretory IgM and IgA have been found to exist in a polymeric state in the absence of J chain (3, 4), and polymerization of monomers can occur independently both in vivo (5) and in vitro (6, 7). J chain synthesis has been detected in plasma cells producing IgG or light (L)[1] chains only, as well as in variants selected to be defective in heavy (H) and/or L chain synthesis (8–12). Immunofluorescence studies have shown that J chain synthesis increases parallel to Ig synthesis as lymphocytes are stimulated by mitogens (13). Although some investigators have reported that resting, surface Ig^+ B lymphocytes are negative, recent evidence with more sensitive immunocytochemical techniques indicates that they, too, contain J chain (14). These observations suggest that J chain biosynthesis is not solely linked to the secretion of polymeric immunoglobulins by plasma cells.

Recently, a number of unusual human cell lines have been derived in this laboratory from patients with various disorders (15, 16). These cell lines, which phenotypically resemble precursors in the B cell lineage, are readily amenable to the analysis of events surrounding immunoglobulin synthesis and the ontogeny thereof. To identify more precisely the point at which the function of J chain becomes essential in this differentiation process, we have used a variety of techniques, both in vivo and in vitro, to assay for its presence within these lines.

Materials and Methods

Antisera. Antibodies to human IgM and κ L chains were raised in rabbits against purified myeloma proteins and affinity-selected as described elsewhere (15). One rabbit antiserum to human J chain was obtained from Dr. J. Heremans, and three different rabbit antisera to human J chain (antisera 22, 271 and 321) were kindly provided by Dr. J. Mestecky. One of

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[1] *Abbreviations used in this paper:* EB, Epstein-Barr; EBNA, EB virus nuclear antigen; H, heavy; L, light; mRNA, messenger RNA; NTET, 150 mM NaCl; 20 mM Tris-HCl, pH 7.6; 10 mM EDTA, 1% Trasylol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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these, antiserum 22, used for the immunoprecipitations presented in Figs. 1 and 2, has been tested by radioimmunoassay and found to be nonreactive to μ, α, γ, λ, or κ determinants (W. H. Kutteh, University of Alabama, personal communication). One additional rabbit anti-human J chain antiserum was raised in this laboratory. All antisera were titrated within the immunoprecipitation systems described below and checked for specificity at this detection limit by cold inhibition with purified proteins (Results).

Cells and Culture Conditions. Lymphoblastoid cell lines of different characteristics were chosen for study. All were positive for the Epstein-Barr (EB) virus nuclear antigen (EBNA) and for Ia antigens, and negative for markers of the T, myeloid, or erythroid series. Most synthesized H and/or L chains (see Table I). Representative of a unique group of cell lines were Josh 7a, KLM 2, and Josh 7b. These lines, termed round cell lines, are distinguished from previously described B lymphoblastoid lines by the absence of detectable Ig synthesis and by special growth and morphologic characteristics. They are related to the B cell lineage by surface marker analysis and possibly represent some type of B precursor cell (15). Josh 4, like Josh 7 and its subclones, was derived from the bone marrow of a patient with X-linked agammaglobulinemia and resembles a pre-B cell in its expression of only cytoplasmic μ chains (16). Daudi (a Burkitt's lymphoma line), SeD (a chronic lymphocytic leukemia line), and 32a.1 and RPMI-8866P (B lymphoblastoid lines) express various classes of surface and/or secretory Ig; the B line Raji was also studied. Other human cell lines used included the T cell lines Jurkat, CCRF-CEM, and KE37; the myeloid lines HL-60 and ML-1; and the monocyte-like line U937. All cell lines were maintained in suspension cultures as described previously (15).

Before pulse labeling with [^{35}S]methionine (700 Ci/mmol) (New England Nuclear, Boston, Mass.), cells were washed twice in phosphate-buffered saline (PBS), resuspended at a concentration of 4×10^6 cells/ml in RPMI-1640 medium (Microbiogical Associates Bioproducts, Walkersville, Md.) that lacked methionine and was supplemented with 5% dialyzed agammaglobulinemic horse serum (Grand Island Biological Co., Grand Island N. Y.), and cultured for 2 h at 37°C. Cells were then pelleted, resuspended in 0.1 ml of the same medium, and pulsed with 500 μCi/ml of [^{35}S]methionine for 8 min at 37°C. To follow labeled products in chase conditions, cells pulsed in this manner were resuspended to a concentration of 1×10^6 cells/ml in RPMI-1640 that contained methionine, supplemented with 5% agammaglobulinemic horse serum, and incubated at 37°C for various time periods.

Labeled cells were washed twice in ice-cold PBS containing 2 mM methionine and then lysed in NTET buffer (150 mM NaCl; 20 mM Tris, pH 7.6; 10 mM EDTA; 1% Trasylol) (FBA Pharmaceuticals, Inc., New York) that contained 1% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.), 10 mM *N*-ethylmaleimide, and 0.5 μg/ml pepstatin (1 ml/10^7 cells). After 20 min on ice with intermittent vortexing, a postnuclear supernate was prepared by centrifugation at 10,000 rpm for 10 min at 4°C.

In Vitro Translation. Total cellular RNA was prepared from frozen pellets of cells grown in mass culture, by an SDS-phenol/chloroform/isoamylalcohol-proteinase K procedure described elsewhere (17). The LiCl-precipitated RNA was washed twice by ethanol precipitation and translated in the presence of [^{35}S]methionine in a wheat germ cell-free system (18) optimized for salts to give efficient translation of J chain (3.8 mM Mg^{+2}, 115 mM KCl). Posttranslationally, the samples were adjusted to the conditions of NTET buffer with 1% Triton X-100, and kept on ice.

Immunoprecipitation. Samples that contained [^{35}S]methionine-labeled polypeptides, generated in vivo or in vitro, were immunoprecipitated immediately after adjustment in the Triton X-100 buffers described above. The use of whole rabbit serum for J chain immunoprecipitations necessitated a preclearance step with 50 μg of normal rabbit serum for 1 h at room temperature, followed by absorption onto Sepharose CL-4B beads coated with sheep anti-rabbit Ig. In preliminary experiments, this preclearance step was found not to precipitate J chain. Thereafter, between 5 and 10 μl of the anti-J chain serum was added to the cleared supernates and incubated for 2 h at room temperature. Immune complexes were absorbed onto sheep anti-rabbit Ig Sepharose for 1 h at 4°C, rotating on a wheel. The Sepharose beads were washed three times in NTET buffer that contained 1% Triton X-100, once in NTET buffer that contained 0.05% sodium dodecyl sulfate (SDS), twice in NTET buffer that contained no detergents, and then resuspended in a polyacrylamide gel electrophoresis loading buffer (2.5%

SDS, 20% sucrose, 0.008% bromphenol blue, 80 mM Tris-HCl, pH 7, and 50 mM dithiothreitol). After 30 min at 37°C, samples were incubated at 100°C for 3 min, and then alkylated in the presence of 250 mM iodoacetamide for 30 min at 37°C.

Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described (17). 7.5–15% gradients best resolved intermediates in the biosynthesis of J chain, but 10% gels served better the resolution of both J and μ chains. All gels were run at 25 mA, constant current.

For analysis of chains by alkaline-urea electrophoresis, samples were first run on 7.5–15% SDS gels, which were subsequently radioautographed without fixing. Bands corresponding to J chain in relative mobility were cut out of this first gel and applied to the top of alkaline-urea slab gels, prepared as described (19). These gels were then processed for fluorography (20).

Peptide Maps. [^{35}S]Methionine-labeled J chains resolved on unfixed 7.5–15% SDS gels were identified by radioautography and peptide mapped by the procedure of Cleveland et al. (21). Details and modifications are outlined in the legend to Fig. 3.

Results

The characteristics of the cell lines used in this study are presented in Table I. All are positive for EBNA and for Ia. Most can be staged into provisional levels of B cell differentiation by the criteria of immunoglobulin expression. Josh 4 phenotypically resembles a pre-B cell line in its expression of only cytoplasmic μ chains. Daudi, SeD, RPMI-8866P, and 32a.1 represent increasingly differentiated phenotypes, ranging from the expression of only surface IgM (Daudi) to the display of an H chain switch in the cases of RPMI-8866P (γ) and 32a.1 (α). The round cell lines Josh 7 and KLM 2 are unique cell types that synthesize no functional immunoglobulins. Their phenotypic markers, however, most closely relate them to the B cell lineage.

J chain biosynthesis in these lines was probed initially by direct immunoprecipitation of [^{35}S]methionine pulse-labeled products. A fluorograph of one such experiment is shown in Fig. 1. As expected, a radiolabeled product comigrating with human J chain was observed in immunoprecipitates from the polymer-secreting lines SeD (IgM; lane 1) and 32a.1 (IgA), from the monomer-secreting line RPMI-8866P (IgG), and from the heterogeneous collection of B cell populations in human tonsil. In addition, however, similar products were found in the Burkitt's lymphoma line Daudi, in the pre-B line Josh 4 (lane 2), and in the round cell lines Josh 7a, KLM 2, Josh 7b (lanes 4–6, respectively). No products comigrating with J chain were immunoprecipitated from the T cell lines KE37 (lane 7), CCRF-CEM, and Jurkat; nor were such

TABLE I
Summary of Primary Cell Lines

Cell line	Cytoplasmic Ig*	Surface Ig*	Secreted Ig‡
Josh 7	—	—	—
KLM 2	—	—	—
Josh 4	μ	—	—
Daudi	—	μ,κ	—
SeD	μ,κ	δ,μ,κ	μ,κ
RPMI-8866P	γ,κ	γ,κ	γ,κ
32.a.1	α,λ	α,λ	α,λ

* Detected by immunofluorescence.
‡ Detected by hemagglutination-inhibition and pulse-chase experiments with [^{35}S]methionine.

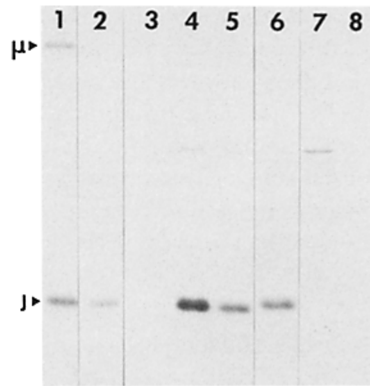

FIG. 1. In vivo biosynthesis of J chain by B and precursor cells: SeD (lane 1), Josh 4 (lane 2), Josh 7a (lane 4), KLM 2 (lane 5), and Josh 7b (lane 6). Cells were pulsed with [^{35}S]methionine for 30 min, immunoprecipitated with rabbit anti-human J chain antiserum, and processed for 10% SDS-PAGE as described in Materials and Methods. Specificity controls include cold competition of the labeled product from Josh 4 with unlabeled J chain (lane 3) and the absence of labeled immunoprecipitates from the non-B cell lines KE37 (lane 7) and U937 (lane 8). The relative mobilities of the μ H chain and native J chain are indicated on the left.

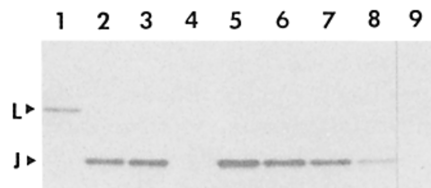

FIG. 2. In vitro biosynthesis of J chain by B and precursor cells: tonsillar B cells (lane 2), Daudi (lane 3), Josh 4 (lane 5), Josh 7a (lane 6), KLM 2 (lane 7), and Josh 7b (lane 8). Products were synthesized in a wheat germ cell-free system from total cellular RNA, immunoprecipitated with rabbit anti-human J chain antiserum, and processed for 7.5–15% SDS-PAGE. Lane 4: cold competition of the product from Daudi with native human J chain; lane 9: immunoprecipitate from the T cell line KE37; lane 1: relative mobility of the primary translation product of the L chain from SeD.

products observed in the cell lines of nonlymphoid origin U937 (lane 8), ML-1, and HL-60. These results have been repeated with five different anti-J chain antisera under a variety of pulse-chase conditions with no qualitative difference.

To better define the J chain-like products synthesized in the pre-B and round cell lines, an analysis was undertaken of their primary translation products, which were synthesized in vitro in a wheat germ cell-free system. Under these conditions it was expected that J chain would not: (*a*) comigrate in unglycosylated form with L chains; (*b*) would not associate—covalently or noncovalently—with H chains; and (*c*) would not be degraded or otherwise modified; each of these is a phenomenon that can complicate the further characterization of J chains synthesized in vivo ([19, 22]; cf. Fig. 1, lane 1—μ from SeD is coprecipitated with J). Fig. 2 is a fluorograph of J chain immunoprecipitates from the total translation products of tonsillar B cells and Daudi, Josh 4, Josh 7a, KLM 2, Josh 7b, and KE37 cells. All but KE37 yielded a comigrating product, the precipitation of which, as shown for Daudi (lanes 3 and 4), was competitively inhibited by cold, native J; SeD, RPMI-8866P, and 32a.1 yielded a

similar primary translation product. The relative mobility of the κ L chain from SeD in such a translation system is included for comparison (lane 1).

Two additional approaches demonstrated that these primary translation products were indistinguishable from the primary translation product of J chain found in tonsillar B cells. First, all were found to comigrate, at an acidic isoelectric point, with native J chain by alkaline-urea gel electrophoresis. Second, limited proteolysis with *Staphylococcus aureus* V8 protease generated identical peptide maps from each. Results of such an experiment are shown in Fig. 3 for J chains derived from the tonsillar B cells, Josh 4, and KLM 2.

Discussion

J chain has previously been detected by a variety of techniques in cells actively synthesizing IgM, IgG, and IgA for secretion (8, 9, 12, 13), in myeloma variants selected for the loss of H and/or L chain synthesis (10, 11), and within the rough endoplasmic reticulum of B cells bearing surface Ig (14). The present study confirms these findings and extends them to an analysis of the primary translation product of J chain within each of these and other cell types. In accordance with the findings of previous studies, B cells that have differentiated to the point of synthesizing monomeric immunoglobulin of any class were found to synthesize J chain. New, and apparently at variance with the studies in the mouse system, is the finding of J chain synthesis in B lineage cells before this stage.

The above data indicate that J chain is synthesized in a series of unique human cell lines, phenotypically similar to B cell precursors. Present in each is a messenger RNA (mRNA) species that, in vitro, encodes the synthesis of a product indistinguishable in serologic characteristics, in relative mobility on SDS-PAGE and on alkaline-urea gel electrophoresis, and in peptide map from J chain derived from more differentiated B cells. In vivo, this mRNA is a functional species, yielding products similar to native

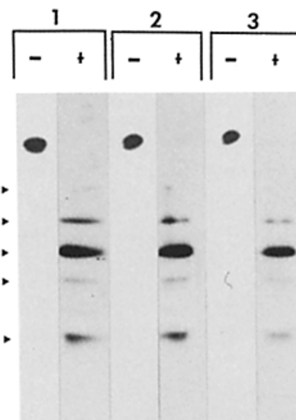

FIG. 3. Peptide maps of J chain primary translation products from (1) tonsillar B cells, (2) Josh 4, and (3) KLM 2. Products were synthesized in vitro, immunoprecipitated with rabbit anti-human J chain antiserum, and subjected to electrophoresis on a 7.5-15% SDS-PAGE. Bands corresponding to J chain were excised from this unfixed gel and again subjected to electrophoresis on a 15% polyacrylamide gel in the absence of (−) or presence (+) of 1 μg/ml *Staphylococcus aureus* V8 protease. Digestion within the gel proceeded for 30 min at room temperature, at which point the peptides were resolved. Arrows on the left indicate five [^{35}S]methionyl peptides shared.

J chain in pulse-chase experiments. In vivo and in vitro, this product was found only in lines of proven or putative B cell origin and not in lines with T or myeloid characteristics. The finding of J chain in these lines is somewhat surprising in view of previous reports. This may be reflective of a difference in the sensitivities of the assays used here and those used in the past (in particular, immunofluorescence [12]), of a species difference in the expression of J chain, or of the character of the cell lines used in this study.

The accumulated evidence suggests, but does not prove, that the cell lines studied here do in fact represent normal precursors to B cells (15, 16). The round cell lines (Josh 7 and KLM 2) are representative of several lines, all with similar growth characteristics and morphology. All are positive for EBNA, Ia antigens, Fc receptors, C3 receptors, and negative for markers of the T, myeloid, or erythroid series. The finding, in this study, that such cells also synthesize J chain more firmly places them into the B cell lineage. That these similar cell types were derived from different patients with several disorders, ranging from monocytic leukemia to X-linked agammaglobulinemia, indicates that they are not reflective of any one particular defect. It is likely that the immortalization of such normal precursor cells in these cases was dependent upon the relative deficiency of mature B cells in these patients which ordinarily overgrow the cultures. This is evident in the case of Josh 7, derived from the bone marrow of a patient with X-linked agammaglobulinemia who had few Ig^+ cells. The derivation of Josh 4 from the bone marrow of the same patient adds further support to this thesis. This line, in its expression of only cytoplasmic μ chain, phenotypically fits the description of a normal pre-B cell.

The ability to detect J chain in such cells was dependent upon their availability as continuously growing lines, as well as upon the sensitivity of the techniques used herein. Immunofluorescence does not so clearly demonstrate the presence of J chain as does either radiolabeling of cells in vivo or immunoperoxidase staining *in situ.* Given the dynamic state of J chain in vivo (vis-à-vis chain association, glycosylation, and degradation), even the results of radiolabeling can be ambiguous. A negative result by any of the above assays is, consequently, difficult to interpret. Little ambiguity exists in the analysis of the total translation products synthesized in vitro from these cells: mRNA for J chain is present, translatable, and (coupled with pulse-chase studies) found to be functional in vivo. It is likely that, if studied by such a method, other B cell types once thought deficient will be found, in fact, to synthesize J chain.

Recent studies in the mouse (23) with a transformed cell line with pre-B cell characteristics indicated that J chain was not synthesized and not translated in vitro. These results are difficult to relate to those of the present studies and the explanation of the difference is not apparent. It is conceivable that the EB virus lines utilized in this work do not reflect the normal situation. This possibly could apply to the round cell lines that showed J chain translation in vitro without Ig translation. These lines could be aberrant types and not precursors, although they were obtained from the bone marrow of widely divergent disorders. However, the pre-B cell line appeared typical in all respects and showed J chain synthesis along with μ chain but not light-chain synthesis. In addition, the well-known Raji line that has been thought to resemble "pre-B like" cells (24) also showed synthesis of J chain. It was also evident that the Daudi line, which has membrane Ig but does not secrete, synthesized J chain.

The exact stage in the hierarchy of gene activation during B cell ontogeny at which J chain synthesis begins remains to be determined but the present results indicate that it is before secretion. There is no question about the marked increase in J chain synthesis at the later period when Ig synthesis increases and secretion begins (13).

Irrespective of the final resolution of this question, the detection of J chain synthesis in the lines without Ig synthesis aided in relating them to cells of B cell lineage.

Summary

Human cell lines that resemble precursors in the B cell lineage have been found to synthesize J chain. In vivo pulse labeling, together with in vitro translation of total cellular RNA in a wheat germ cell-free system, detected the synthesis of J chain in immunoglobulin-secreting cell lines, in a cell line with only surface IgM, as well as in the pre-B-like cell line Josh 4 and the round cell lines Josh 7 and KLM 2. The primary translation products of J chain from all of these cell lines were found to be indistinguishable from one another by serologic criteria, by relative mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, by charge as judged by alkaline-urea gel electrophoresis, and by peptide mapping. These findings suggest that the onset of J chain biosynthesis represents a relatively early event in B cell ontogeny, occurring before the development of immunoglobulin polymer-secreting cells. Its role may, consequently, be fundamental to the biosynthesis of all immunoglobulins, at different stages of B cell differentiation.

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References

1. Inman, F. P., and J. Mestecky. 1974. The J chain of polymeric immunoglobulins. *Contemp. Top. Mol. Immunol.* **3:**111.
2. Koshland, M. E. 1975. Structure and function of the J chain. *Adv. Immunol.* **20:**41.
3. Mihaesco, C., E. Mihaesco, and H. Metzger. 1973. Variable J-chain content in human IgM. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **37:**303.
4. Tomasi, T. B., and D. S. Czerwinski. 1976. Naturally occurring polymers of IgA lacking J chain. *Scand. J. Immunol.* **5:**647.
5. Stott, D. I. 1976. Biosynthesis and assembly of IgM. Addition of J chain to intracellular pools of 8S and 19S IgM. *Immunochemistry.* **13:**157.
6. Kownatzki, E. 1973. Reassociation of IgM subunits in the presence and absence of J chain. *Immunol. Commun.* **2:**105.
7. Eskeland, T. 1974. IgM reassociation in vitro: no influence of J chain on the amount of polymers. *Scand. J. Immunol.* **3:**757.
8. Kaji, H., and R. M. E. Parkhouse. 1974. Intracellular J chain in mouse plasmacytomas secreting IgA, IgM and IgG. *Nature (Lond).* **249:**45.
9. Brandtzaeg, P. 1974. Presence of J chain in human immunocytes containing various immunoglobulin classes. *Nature (Lond).* **252:**418.
10. Kaji, H., and R. M. E. Parkhouse. 1975. Control of J chain biosynthesis in relation to heavy and light chain synthesis, polymerization and secretion. *J. Immunol.* **114:**1218.

11. Mosmann, T., and R. Baumal 1975. Synthesis but not secretion of J chain by variant mouse myeloma cells which lose α-chain-synthesizing ability. *J. Immunol.* **115:**955.
12. Mestecky, J., J.-L. Preud'homme, S. S. Crago, E. Mihaesco, J. T. Prchal, and A. J. Okos. 1980. Presence of J chain in human lymphoid cells. *Clin. Exp. Immunol.* **39:**371.
13. Mestecky, J., R. J. Winchester, T. Hoffman, and H. G. Kunkel. 1977. Parallel synthesis of immunoglobulins and J chain in pokeweed mitogen-stimulated normal cells and in lymphoblastoid cell lines. *J. Exp. Med.* **145:**760.
14. Nagura, H., P. Brandtzaeg, P. K. Nakane, and W. R. Brown. 1979. Ultrastructural localization of J chain in human intestinal mucosa. *J. Immunol.* **123:**1044.
15. Fu, S. M., and J. N. Hurley. 1979. Human cell lines containing Epstein-Barr virus but distinct from the common B cell lymphoblastoid lines. *Proc. Natl. Acad. Sci. U. S. A.* **76:**6637.
16. Fu, S. M., J. N. Hurley, J. M. McCune, H. G. Kunkel, and R. A. Good. 1980. Pre-B cells and other possible precursor lymphoid cell lines derived from patients with X-linked agammaglobulinemia. *J. Exp. Med.* **152:**1519.
17. Erickson, A. H., and G. Blobel. 1979. Early events in the biosynthesis of the lysosomal enzyme cathepsin D. *J. Biol. Chem.* **254:**11771.
18. Dobberstein, B., and G. Blobel. 1977. Functional interaction of plant ribosomes with animal microsomal membranes. *Biochem. Biophys. Res. Commun.* **74:**1675.
19. Mosmann, T. R., Y. Gravel, A. R. Williamson, and R. Baumal. 1978. Modification and fate of J chain in myeloma cells in the presence and absence of polymeric immunoglobulin secretion. *Eur. J. Immunol.* **8:**94.
20. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46:**83.
21. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252:**1102.
22. Wilde, C. E., and M. E. Koshland. 1973. Molecular size and shape of the J chain from polymeric immunoglobulins. *Biochemistry.* **12:**3218.
23. Mather, E. L., F. W. Alt, A. L. M. Bothwell, D. Baltimore, and M. E. Koshland. 1981. Expression of J chain RNA in cell lines representing different stages of B lymphocyte differentiation. *Cell.* **23:**369.
24. Preud'homme, J. L., M. F. Gourdin, F. Reyes, and M. Fellous. 1978. Human lymphoid cell lines with pre-B cell characteristics. *In* Human Lymphocyte Differentiation: Its Application to Cancer. B. Serrou and C. Rosenfeld, editors. Elsevier North-Holland, Inc., New York. 345.