Brief Definitive Report

A MURINE HYBRIDOMA WITH LARGE CYTOPLASMIC INCLUSIONS OF κ LIGHT CHAINS

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There have been several case studies in the literature of patients with lymphoproliferative disorders in which inclusion bodies were seen in the cytoplasm of malignant cells. The nature of these structures has remained elusive, although in a few cases they appear to be related to immunoglobulins or Ig subcomponents. This report details the establishment of a murine hybridoma that forms intracytoplasmic inclusion bodies similar in some respects to those described in human patients. Preliminary immunochemical analysis suggests a structure composed of polymerized or crystallized Ig L chains.

Materials and Methods

Hybridomas. Spleen cells from a BALB/c mouse immunized intravenously 4 d previously with ARS coupled to *Brucella abortus* (1) were fused with the nonsecreting myeloma cell line P3X63-Ag8.653 using standard hybridization protocols (2). Cells were grown in RPMI 1640 with 5×10^{-5} M 2-ME and 10% FCS.

Fluorescent Antibody Staining. Goat antibodies specific for H and L chain isotypes (Southern Biotechnology Associates Inc., Birmingham, AL) were modified with biotin (3). Briefly, antibodies (1 mg/ml) were dialyzed overnight against borate buffer (0.05 M borate, 0.15 M NaCl, pH 8.5) at 4°C followed by reaction for 4 h at room temperature (RT) with 50 μl biotin-*n*-hydroxysuccinimide (1 mg/ml in DMSO) per mg antibody. Antibodies were then dialyzed against PBS with azide (0.05%). Viable cells for cytoplasmic staining were separated on Ficoll-Hypaque (4), washed, spread on cleaned microscope slides, allowed to dry, and fixed in 95% ethanol, 5% acetic acid in a dry ice–ethanol bath for 15 min. Slides were stained with biotinated antibodies and avidin-FITC.

Inclusion Body Purification. Viable cells were isolated on Ficoll-Hypaque, washed in serum-free medium, and lysed in a minimal volume of 0.4% NP-40 for 10 min at RT. The lysate was mixed with isotonic Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), and centrifuged at 15,000 *g* for 15 min. Relative densities were determined from a tube loaded with density marker beads (Pharmacia Fine Chemicals). Isolated inclusion bodies formed a discrete band that was washed free of Percoll with a low-speed spin.

SDS-PAGE and Western Blots. Purified inclusion structures were dissolved by boiling for 5 min in reducing or nonreducing sample buffer. Low molecular weight standards (Bio-Rad Laboratories, Richmond, CA), MOPC-315 (IgA, λ) and MOPC-21 (IgG1, κ)-purified proteins were also run. Samples were electrophoresed in 10% polyacrylamide gels at 12 mA per gel (5). After electrophoresis, the gel was cut into sections, with identical lanes of references and sample. One section was stained with Coomassie Brilliant Blue. Other sections were equilibrated in transfer buffer (384 mM glycine, 50 mM Tris, 20% methanol, pH 8.3) and the separated proteins were transferred electrophoretically overnight into a nitrocellulose membrane using a Transblot chamber (Bio-Rad Laboratories).

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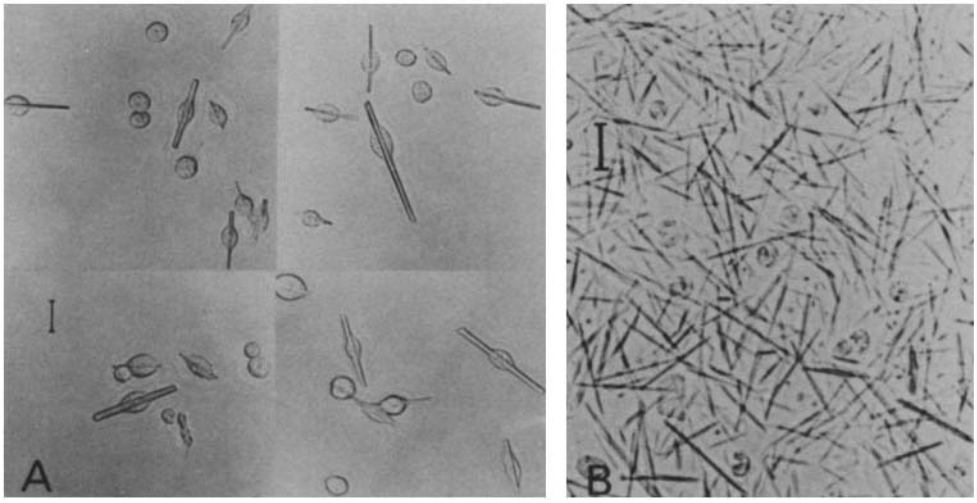

FIGURE 1. Phase-contrast photomicrographs of (*A*) viable cells isolated from 12-d-old cultures (× 200) and (*B*) inclusion bodies isolated from 12-d-old cultures (× 300). Bars represent 20 μm.

Membranes were dried, incubated for 1 h at RT in blocking solution (20 mM Tris, 150 mM NaCl, 3% gelatin, 2.5% BSA, pH 7.2), and probed with a biotinated antibody specific for either H or L chains. Bound antibodies were developed with avidin-peroxidase (No. 3151; Sigma Chemical Co., St. Louis, MO) and 4-chloro-1-napthol (Bio-Rad Laboratories). All intermediate washings were done with Tris-buffered saline (TBS) (20 mM Tris, 150 mM NaCl, pH 7.2). Gels were also stained with dansyl hydrazine to visualize glycoproteins (6).

Results

The hybridoma techniques used involve plating the fusion products at low multiplicities directly into 96-well plates with feeder layers to effect cloning directly (2). During microscopic examination 10 d after fusion, 1 of 54 clones was noted in which a small percentage of the cells contained large cytoplasmic inclusions. No ARS-specific antibody was detected in the supernatant. The cell line was cloned twice by limiting dilution, but all subclones remained morphologically heterogeneous, with only a small percentage of cells having inclusions. The number increased, however, as cultures aged and ceased rapid division. The percentage of cells with inclusions in cultures maintained in log-phase growth by frequent passage rarely exceeded 0.1%. In 10–14 d cultures, as many as 40% of viable cells contained cytoplasmic inclusions.

Representative examples of a subclone (F10) are shown in Fig. 1*A*. The intracytoplasmic structures are invariably linear and crystal-like, and are usually present as a single inclusion that bisects the cell. Structures vary in size up to 90 μM in length, often grossly distorting normal cellular morphology. It appears that the cell membrane surrounds the entire structure, but this is difficult to verify in extreme cases. At cell death, inclusions often disintegrate into several fibrils of approximately the same length as the original structure, suggesting a linear subunit composition. Although the morphology illustrated in Fig. 1 is typical of >95% of cells with inclusions, there are also cells with more chaotic

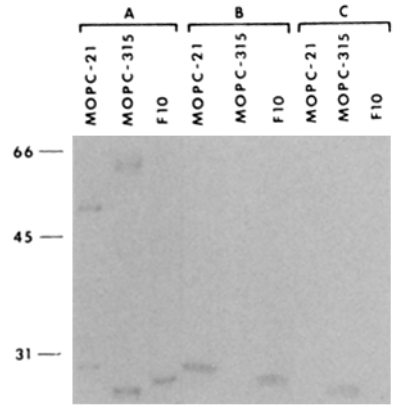

FIGURE 2. Electrophoretic and immunoblot analysis of material solubilized from inclusion bodies. Purified MOPC-21 (IgG1, κ), MOPC-315 (IgA, λ), and material solubilized from isolated inclusion bodies (F10) were reduced and analyzed in 10% gels. (*A*) Stained directly with Coomassie Brilliant Blue; (*B* and *C*) transferred to nitrocellulose and probed with antibodies specific for κ (*B*) or λ (*C*) L chains. $M_r \times 10^{-3}$ are shown.

structures, including cells with multiple intersecting inclusions. It has not been possible to establish subclones in which the frequency of the various morphological types varies from that described above.

Lysis of F10 cells in 0.4% NP-40 followed by Percoll purification yields a discrete band with minimal cellular contamination at a relative buoyant density of 1.158 g/ml containing structures microscopically identical to those seen in the cells (Fig. 1*B*). Such preparations, washed in PBS, were used for SDS-PAGE and Western analyses. Coomassie staining (Fig. 2*A*) revealed the expected H and L chain bands in the Ig controls. Material from the F10 cytoplasmic inclusion bodies migrated as a single band intermediate between the κ and λ chains, with an M_r of ~26,000. Nonreducing gels showed an additional band of ~45,000 (not shown). When reduced proteins transferred to the nitrocellulose membrane are probed with an anti-κ serum, the MOPC-21 L chain and the F10 inclusion material both bind the antibody (Fig. 2*B*). In nonreduced samples, the 45,000 M_r material also binds κ-specific antisera. When a similar Western blot is probed with a λ-specific antibody (*C*) only the MOPC-315 L chain binds the indicator. H chain–specific antisera stain only the appropriate H chains (not shown).

A similar SDS-PAGE gel was stained with dansyl hydrazine, a fluorescent reagent that stains glycoproteins (6). No fluorescence was seen in association with the 26,000 M_r F10 protein, whereas glycosylated MOPC-21 and MOPC-315 H chains stained (not shown). The combined data therefore suggest that the material solubilized from the inclusion bodies is a mixture of monomers and dimers of a nonglycosylated protein of 26,000 M_r antigenically similar to κ chain.

Slide preparations of F10 cells were stained with antibodies specific for various H and L chains. Inclusion bodies stained brightly with biotinated anti-κ antibody and avidin FITC (Fig. 3). There was no staining with antibodies specific for λ chains or H chain isotypes. This indicates that the isolated material analyzed by SDS-PAGE is in fact associated with the cytoplasmic inclusions within viable cells.

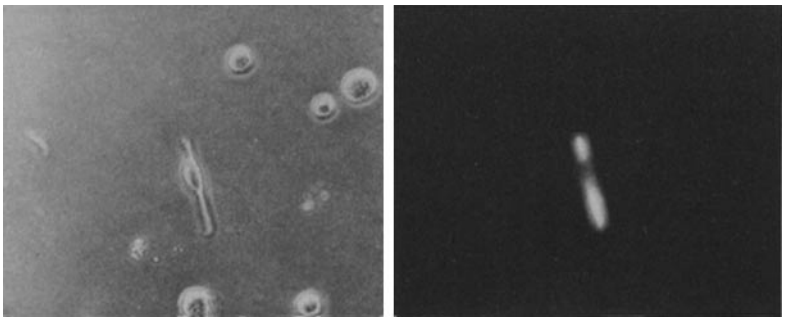

FIGURE 3. Immunofluorescence of fixed cells stained with antisera specific for κ light chains. Same field illuminated with white light (*left*) or UV (*right*) × 200.

Discussion

The data presented here are consistent with the retention and intracellular deposition of complete κ chain protein. The mechanism by which a normally secreted, globular protein is assembled into these large inclusions is unclear. Defects in glycosylation lead to the accumulation of large amounts of Ig in the cytoplasm of plasmacytomas, but inclusion bodies are not formed (7). Amino acid substitutions that alter tertiary conformation in proteins destined for export result in accumulation of the product in transfected L cells, but without aggregation into recognizable structures (8). One possible mechanism may be the inability to enzymatically remove the leader sequence normally cleaved from the NH_2-terminal end of L chain protein. Such removal is a prerequisite for secretion (9), and failure to do so could lead to accumulation in the cytoplasm. The hydrophobicity of the leader sequence may also force a partitioning of the protein from the aqueous environment of the cytoplasm, thus accounting for self-assembly into the observed structures. The inability to cleave the leader could either be due to a unique characteristic of the κ chain sequence, or to a defect in the signal peptidase system. Alternatively, it could be postulated that the hydrophobicity arises from the use of a V region rich in hydrophobic residues. Sequencing studies we have initiated should differentiate these hypotheses.

Fluorescent staining demonstrates that κ protein is associated with the inclusion body, but does not imply that it is the sole component. Percoll sedimentation however, allows the purification of the structure to virtual homogeneity dependent solely on its buoyant density. Structures isolated in this manner appear microscopically identical to those in the cytoplasm of intact cells, and yield a single protein band on SDS-PAGE of ~26,000 M_r that reacts strongly with κ-specific antisera, suggesting that the κ chain is the major if not the only protein present. Other components may be present at levels below detection limits, or be of a nonprotein, noncarbohydrate nature.

The nature of the inclusions and the mechanism responsible for their deposition may be relevant to two pathological states described in humans. There have been several reports of both malignant and nonmalignant lymphoproliferative disorders in which the proliferating cells contain crystalline cytoplasmic inclusions (10–14). These individual case descriptions share several characteristics. Inclusions are usually seen in slides of bone marrow, lymph node, or other lymphoid

tissues. They appear as rod- or needle-like structures of variable length, in some cases extending outside the cell. Clinical data often but not always indicate a monoclonal gammopathy and/or Bence-Jones proteins. Inclusions are always associated with cells making single isotypes of H and L chains (i.e., monoclonal), but usually do not stain with fluorescent anti-Ig. In two cases (10, 11), however, they stained with reagents specific for the relevant H or L chain. A single attempt to establish a cell line with inclusions using lectin-stimulated cells was unsuccessful (12). The cell line described here is similar in its lymphoid origin, monoclonality, and morphology to the cells seen in these patients, and may provide a useful in vitro model through which the mechanism of inclusion formation can be elucidated.

The extracellular deposition of fibrillar L chain protein is etiologic in one form of systemic amyloidosis (15–18). Since the M_r of the L protein associated with these deposits is ~11,000, it is not an entire L chain (18). Amino acid sequence determinations suggest that the deposits are composed of polymerized L chain variable regions (15). The in vitro formation of such structures from Bence-Jones proteins has also been shown to require proteolysis of the intact molecule (16). From such data, a concept of amyloidogenesis has arisen requiring both the overproduction and subsequent proteolytic modification of a precursor protein. The deposition of structures composed of intact L chain as described here shows that proteolysis is not an absolute precondition for homologous association and deposition and raises the possibility of an amyloidogenic sequence in the primary structure of the κ light chain molecule. The investigation of such a sequence should be greatly facilitated by this cell line.

Summary

A murine hybridoma cell line has been established that consistently forms large cytoplasmic inclusions. These structures bind antibody specific for mouse κ L chain when stained in situ. SDS-PAGE analysis of isolated inclusion bodies produce a single protein band of ~26,000 M_r that reacts with anti-κ antibody when transferred to nitrocellulose. No carbohydrate was detected in association with the purified protein. These data are consistent with the intracellular retention and deposition of complete κ L chain protein.

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