Gamma heavy chain disease in man: Translation and partial purification of mRNA coding for the deleted protein*

(immunoglobulins/deletions/glycosylation)

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ABSTRACT Lymphoid cells obtained from the peripheral blood of a patient with heavy chain disease have been established in long-term culture. They continue to produce a protein antigenically identical to the deleted γ3 heavy chain disease protein found in the patient’s serum. The availability of the cell line has made it possible to analyze the mRNA coding for this protein. The primary in vitro translation product is 1500-2000 daltons larger than the polypeptide portion of the cytoplasmic or secreted protein and has methionine at the amino terminus. The mRNA sediments at 15.5 S on sucrose gradients and therefore appears to be smaller than the 175 message coding for normal-sized mouse γ2 chains. It contains a base sequence that codes for a hydrophobic amino-terminal peptide not found in the cytoplasmic protein. There was no evidence for the synthesis of translatable light chain message by these cells. The present data suggest that this protein results from a primary somatic genetic event that gave rise to a cell product bearing a normal amino terminus sensitive to limited proteolytic digestion. The serum protein thus appears to begin in the hinge region but, in fact, contains a normal heavy chain initiation site.

Each immunoglobulin polypeptide chain is coded for by two genes that are spatially separated in the genome (1). The mechanism whereby the two discrete genetic elements are integrated to allow their expression as a single polypeptide has not yet been defined. Human heavy chain diseases (HCDs) comprise a class of lymphoproliferative disorders in which the malignant cells synthesize and secrete internally deleted heavy chain (H chain) molecules (2). Detailed chemical analyses of some of the γ proteins have indicated that the molecules had normal NH2-terminal sequences followed by clearly defined deletions beginning at various points within the variable (V) region and extending through the first domain of the constant region (CH1). No normal sequence was usually resumed at the beginning of the hinge and thereafter continued to the COOH terminus with no further aberrations (3, 4). In addition to the defective H chains, most cells producing γ and α HCD proteins also fail to synthesize light chains (L chains).

Structural analysis of the γ3 HCD protein isolated from the serum of patient OMM revealed a monomeric molecular weight of 40,000, an unblocked NH2 terminus, and no associated L chains (5). The NH2 terminal sequence corresponded to the beginning of the normal γ3 hinge. The OMM protein from serum therefore was apparently initiated where normal sequence resumes in internally deleted HCD proteins. Because the deletion was NH2 terminal, encompassing the entire V and CH1 regions, it could have occurred by either partial proteolysis of an abnormal gene product or posttranslational degradation of a normal γ chain.

A permanent cell line was established from the patient’s peripheral blood (6). The cultured line and its subclones synthesized and secreted a protein with the antigenicity and electrophoretic mobility [in sodium dodecyl sulfate (NaDodSO4)/acylamide gels] of the isolated serum protein. There was no evidence for synthesis of either intact heavy or light chains. However, preliminary chemical analysis of the protein isolated from the cytoplasm and secretions of the cultured cells suggested that the primary deleted gene product may have undergone limited proteolysis in vivo (unpublished data). The present experiments were designed to determine the size of the message coding for the OMM protein and the presence or absence of translatable L-chain mRNA.

MATERIALS AND METHODS

Maintenance of the OMM cultured cells, preparation of radioactively labeled cytoplasm and secreted material for immunologic precipitation, and electrophoretic procedures were performed as described (6).

Cytoplasm, material secreted by the OMM cells, and the wheat germ translation mixtures were precipitated by the addition of carrier antigen and specific antiserum in 2-fold antibody excess. The precipitates were extensively washed and dissolved in sample buffer (2% NaDodSO4/0.5% Nonidet P40/0.08 M Tris, pH 7.4). Samples were boiled for 1 min, and an aliquot of each was reduced with 5 mM dithiothreitol for 30 min at 37° followed by alklylation with 10 mM iodoacetamide.

A protein-synthesizing system was prepared from raw wheat germ (General Mills, Kansas City mill, MO) and stored at 4° under vacuum. The methods of Roberts and Paterson (7), with some modifications, were used for the preparation of the lysate and the translation mixture. Unless specified otherwise, the standard protein synthesis mixture contained 100-125 mM KCl, 1.8 mM MgOAc2, 0.3 mM spermidine, 40 μg of creatine kinase per ml, 1 mM ATP, 20 μM GTP, 2 mM dithiothreitol, 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes) (pH 7.6), 100-200 μCi of [35S]methionine per ml, and 20 A260 units of wheat germ lysate per ml plus amounts of RNA as indicated. No unlabeled amino acids were added. The reaction was incubated for 90 min at 30°. Prior to analysis, the mixture was treated with RNase A (20 μg/ml) for 10 min and then boiled for 1 min in 2% NaDodSO4. The protein-synthesizing activity of the mixture was determined by precipitating 2.5-μl

Abbreviations: HCD, heavy chain disease; H chain, heavy chain; V, variable; L chain, light chain; CH1, first domain of the constant region; CH2, second domain of the constant region; OMM, patient with HCD; NaDodSO4, sodium dodecyl sulfate; OMMTr, primary translation product.

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samples in trichloroacetic acid followed by assay of the radioactive precipitates in a scintillation counter (8).

Immunological precipitates were prepared from the translation mixture after adjustment of the samples to 0.5% Nonidet P40 and centrifugation at 8000 × g for 30 min at 4°C in an International PR-J centrifuge. Antibodies and carrier proteins were added to the clarified supernatant as described (6).

For RNA extractions, all glassware and buffer solutions were autoclaved before use. RNA extractions were performed by using either the hot or the cold phenol/NaDodSO4 method (9, 10). Poly(A)-containing RNA molecules were prepared by chromatography on either poly(U)-Sepharose or oligo(dT)-cellulose (11, 12). For gradient separation of various RNA species, the procedures of Cowan et al. were used (13).

RESULTS

When total cytoplasmic RNA, extracted from either cultured cells from clone 45,6,31 of the murine myeloma MPC-11 (an IgG2b, producer) or the OMM cells, was added to the wheat germ lysate, protein synthesis, as measured by the incorporation of [35S]methionine into trichloroacetic acid-precipitable radioactivity, was linear until 10 μg of RNA was added. Amounts larger than this were saturating. Incorporation of radioactivity into the acid-precipitable material was approximately 12-fold greater than when the incubation was carried out in the absence of added RNA.

On addition of 45,6,31 RNA, the wheat germ extract synthesized immunologically precipitable intact H and L chains that behaved similarly, on electrophoresis, to those synthesized by the 45,6,31 cultured cells (Fig. 1). The total RNA extracted from the cloned OMM cells stimulated the synthesis of several proteins by the wheat germ (Fig. 2, lane 2). When the translation mixture was precipitated with antiserum directed against the Fc portion of human γ chain (anti-Fc) and the precipitate was electrophoresed, a single band was seen migrating with an electrophoretic mobility corresponding to a protein with an estimated molecular weight of 38,000 (OMMγ1) (Fig. 2, lane 3). Identical results were obtained when an antiserum raised against an intact γ3 chain (anti-OMMγ3) was used. The antiserum could recognize both the Fc and Fd regions of the γ3 chains. No proteins were immunologically precipitable with an antiserum directed against human F(ab) determinants and capable of reacting with both κ and λ L chains, whether free or bound to any class of H chain (lane 4). Thus, although the wheat germ extract could synthesize complete murine H and L chains when presented with the appropriate mRNA, it synthesized only the γ3 fragment when presented with total cytoplasmic RNA from the OMM cultured cells.

The OMM serum protein contained approximately 7% carbohydrate (5). Because the wheat germ lysate does not glycosylate proteins, it was difficult to compare accurately the size of the OMMγ1 with the polypeptide portion of the OMM protein obtained from the patient’s serum or the cultured cells. Labeled unglycosylated cytoplasmic and secreted proteins were obtained by incubating the cells in the presence of radioactive precursors with and without 2-deoxyglucose, an inhibitor of glycosylation of both viral and immunoglobulin proteins (14, 15). Aliquots of intact OMM cells were incubated with labeled valine, threonine, and leucine or glucosamine in the presence and absence of 2-deoxyglucose. Anti-γ Fc precipitates of cytoplasm were compared with those obtained from the translation mixture on slab gels (Fig. 3). The OMMγ1 (lane 4) migrated slightly faster than the glycosylated cytoplasmic
OMM protein (lane 5) but more slowly than the band "a" protein (lane 3) synthesized in the presence of 2-deoxyglucose. The estimated molecular weight difference between OMMγ, and band "a" is 1500–2000. In lane 3 there was an additional protein band ("b") that comigrated with the glycosylated OMM protein. This band was probably the result of incomplete inhibition of glycosylation by the deoxyglucose (see below).

The anti-γ Fc immunoprecipitates of the glucosamine-labeled cytoplasm are shown in lanes 1 and 2. In the absence of deoxyglucose (lane 2), the radioactive OMM protein migrated to the position occupied by the glycosylated amino acid-labeled molecule. However, in the presence of deoxyglucose (lane 1), the OMM band was not visible, confirming the effectiveness of deoxyglucose in blocking incorporation of the first sugar of the oligosaccharide chain. When the x-ray film was exposed for 23 days, a faint band became visible in lane 1 at the position of the glycosylated protein. As in the amino acid experiments, the block of glycosylation, although considerable, was not absolute.

Lanes 6–9 of Fig. 3 show anti-γ precipitates of the material secreted by the OMM cells in the presence or absence of 2-deoxyglucose. The results were identical to those obtained with cytoplasmic proteins shown in lanes 1, 2, 3, and 5.

The relative sizes of the mRNAs coding for the OMMγ, protein and the 45,6,31 γ and κ chains were determined by cosedimentation of mixed OMM and 45,6,31 total cellular RNA through a 10–40% sucrose gradient (Fig. 4). RNA from each of the eight fractions between the 12S and 18S regions of the gradient were added to the wheat germ lysate in the presence of [35S]methionine and the labeled translation products were analyzed on slab gels (Fig. 5). Lane 1 contained the proteins translated from the total mRNAs extracted from the OMM cells. Lanes 2–7 contained the proteins coded for by messages of decreasing size from the individual gradient fractions. 45,6,31

**DISCUSSION**

The present experiments offer some insight into the genesis of the two defects found in cells producing HCD proteins. The aberrant κ chain could result from the degradation of a normal chain. However, the available amino acid sequence analyses of some of the proteins, indicating the presence of normal NH2 and COOH termini with internal deletions, make simple proteolysis an unlikely explanation. In addition, none of the studies analyzing postribosomal events has identified an intact H chain in the cytosol (16, 17). Some of the γ HCD proteins and most of the κ HCD proteins that have been isolated from the serum or urine seem to be the result of NH2-terminal proteolysis of abnormal chains because they reveal both chemically heterogeneous NH2 termini and internal deletions. The second possible general mechanism involves an abnormality at the level of the genome with or without posttranslational proteolysis. The *in vitro* synthesis of a short γ chain would eliminate degradation as the primary process in the generation of the OMM HCD protein.

It is clear from Fig. 1 that the lysate used in the present experiments is capable of translating both murine and κ L chain.
FIG. 5. Cytoplasmic RNA was prepared from 45,6,31 and OMM cells by the 60° phenol extraction method. Equal amounts of each were mixed and sedimented through a 10-40% sucrose gradient in 0.5% NaDodSO4. Ethanol-precipitated RNA from each fraction was added to the wheat germ lysate in the presence of [35S]methionine. The proteins resulting from the translation of each of the RNA fractions were electrophoresed in a 0.1% NaDodSO4/polyacrylamide slab gel. The positions of the H2L2 (155,000 daltons), H chain (56,000 daltons), and L chain (22,500 daltons) markers are indicated on the left. 18S ribosomal RNA was found in the fraction represented by lane 3. The material found in fraction 10 had a sedimentation coefficient of 12 S. The murine H chain message was primarily in fractions 4 and 5 and that of L chain, in fractions 8 and 9. The translations of OMM mRNA are best seen in lanes 6 and 7. Lane 1 contains the proteins coded for by the total RNA extractable from the OMM cells.

messages (18-20). The use of a human cell line synthesizing an intact γ2 H chain would have been desirable in these experiments; however, such a line was not available for comparison.

The translation of OMM total cytoplasmic RNA yielded a 38,000-dalton protein, OMMγγ, that had the antigenic determinants of the Fc region of a human γ chain (Fig. 2). No additional bands bearing Fd determinants were detectable when the translation mixture was precipitated with various antisera recognizing both Fc and Fd antigens. Similar results were obtained when these antisera were used with labeled lysates of the cultured OMM cells. It therefore was unlikely that a normal-sized H chain was synthesized, even one containing a structural defect that rendered it particularly sensitive to proteolysis at the hinge region. Because the heterologous wheat germ translating system directed the synthesis of the short OMM protein, the defect must have been present in the mRNA and not due to a translation error.

It has been hypothesized that mRNAs of proteins destined for secretion contain a nucleotide sequence coding for a hydrophobic NH2-terminal precursor piece (21). The OMM serum protein had an NH2-terminal deletion. Although preliminary structural analysis suggested that the cytoplasmic protein had an NH2-terminal pyrolidonecarboxylic acid, it comigrated with the serum protein on NaDodSO4 gels. Hence, it probably contained a very small portion of normal V-region sequence. If, in addition, the hydrophobic peptide were present, the protein synthesized by the wheat germ lysate should have been 1000-2000 daltons larger than that found in the intact cells. The molecular weight difference would be obscured, however, by the differences in carbohydrate content between OMMγγ, which had no sugar, and the serum protein. The cellular product was obtained in a sugar-free form by inhibiting glycosylation with 2-deoxyglucose. OMMγγ, migrated slightly faster than the cellular protein synthesized in the absence of 2-deoxyglucose but slower than that produced in the presence of the compound (Fig. 3). The translated protein, therefore, was larger than the unglycosylated cellular protein by 1500-2000 daltons, corresponding to an amino acid extension of 15-20 residues.

Preliminary sequence analysis of OMMγγ identified methionine at the NH2 terminus. In addition, analysis of the [35S]-methionine-containing tryptic peptides of OMMγγ showed a pattern consistent with the presence of an extra, NH2-terminal, methionine-containing peptide.

The results presented here have established that, when translated in a heterologous cell-free system, the OMM mRNA only directs the synthesis of a short protein. The error therefore must be encoded in the cytoplasmic mRNA. We compared the sedimentation of the OMM mRNA with that of a normal-sized murine γ2b, H chain. The results (Figs. 4 and 5) showed that the two messages sedimented in different regions of the gradient. The murine message had an approximate sedimentation coefficient rate of 17 S, whereas that of the OMM molecules was calculated to be 15.5-16 S. Because both molecules had been treated similarly, the differences in sedimentation probably reflected differences in size rather than in secondary structure; hence, the OMM protein appears to be coded by a small mRNA. More precise comparison of the size of the 45,6,31 and OMM mRNAs will determine if the entire size difference can be accounted for by that noted in the coding region.

The OMM protein from serum, in contrast to most of the HCD proteins, does not begin with an NH2-terminal normal V-region sequence; hence, the deletion in the serum protein appeared to be NH2 terminal. Because the OMMγγ contained the precursor piece and the cytoplasmic protein was NH2-terminally blocked, it was comparable to other serum HCD proteins in which internal deletions have been defined. However, in the present case, the genetically deleted H chain has undergone limited postsynthetic proteolysis.

The defect in L-chain production by HCD cells is not readily explained. Because the structural genes for H and L chains are on separate chromosomes (22), it would require two indepen-
dent structural gene abnormalities to account for both the H-chain deletion and the absence of L-chain production. There was no major chromosomal abnormality in the cells; hence, multiple chromosomal loss related to either malignancy or long-term culture was an unlikely explanation. In addition to the HCD protein, the serum of patient OMM contained an intact homogeneous (γδ)2λ2 myeloma protein with apparently normal H and L chains. Thus, the patient did not have a generalized defect in L-chain synthesis (23). Previous experiments established that no L chains could be detected in the cytoplasm or secretions of the OMM cultured cells. The present experiments show that it was not possible to detect the synthesis of any L chains when total cytoplasmic RNA extracted from the OMM cells was added to a cell-free system capable of translating intact L-chain message from a murine or human source (unpublished data). It therefore can be concluded that the OMM cells contained no translatable L-chain message.

The presence of untranslatable L-chain mRNA cannot be excluded. Such a situation has been encountered in β-thalassemia in which β-globin chains were not synthesized in vitro or in the cell-free system using reticulocyte RNA; however, RNA that hybridized with bona fide β-globin cDNA was present (24, 25). Chemical evidence for untranslatable L-chain mRNA has also been obtained when the RNA isolated from a nonproducing murine myeloma line was subjected to oligonucleotide fingerprint analysis and compared to that of previously purified L-chain mRNA (13). Hence, although the OMM cells contain no translatable L-chain mRNA, it remains to be established whether the defect is regulatory rather than structural.

This existence of deletions involving the contiguous polypeptide product of two distinct genes has been invoked to support the notion that the deletion occurs in the DNA (4). Recent data suggest that cytoplasmic mRNAs for adenovirus proteins and rabbit globin, as well as several other eukaryotic proteins, may be produced by nuclear splicing of RNA derived from noncontiguous regions of a larger precursor molecule (26, 27, 28). Such observations imply that aberrant processing of heterogeneous nuclear RNA transcripts could yield deleted proteins. In immunoglobulin-producing cells, a mutational event that is transcribed into the noncoding region of a nuclear RNA precursor could cause a splicing error that results in a short cytoplasmic mRNA containing transcripts of portions of the normal V and C genes.

It is now easier to conceive of an explanation for the relative constancy of the site of resumption of normal H-chain sequence in HCD proteins. The DNA coding for the hinge region may be separated from that coding for the CH1 and CH2 domains by spacer sequences (introns). Deletions encompassing the structural elements may also run into the spacers. It may require a given amount or specific sequence of spacer material preceding a region that codes for structure to allow the expression of that structure. Such a phenomenon could occur at the DNA or precursor RNA level, either case resulting in a short cytoplasmic mRNA.

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