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Gamma heavy chain disease in man: synthesis of a deleted γ_3 immunoglobulin by lymphoid cells in short and long term tissue culture

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SUMMARY

Bone marrow cells were obtained from a patient with gamma heavy chain disease (HCD) whose serum contained a deleted immunoglobulin heavy chain. Incubation of the marrow cells with radioactive amino acids in short term tissue culture resulted in the synthesis of the labelled HCD protein. A permanent cell line was established from the peripheral blood of the patient. Similar labelling studies with the cell line and its cloned progeny demonstrated the synthesis of a protein identical in size and antigenicity to that synthesized by the marrow cells and found in the patient's serum. These experiments clearly demonstrated that, in this case of heavy chain disease, the deleted protein was the synthetic product of a clone of malignant lymphoid cells.

INTRODUCTION

Heavy chain diseases are lymphoproliferative disorders in which the malignant cells produce monoclonal immunoglobulins which are defective with respect to the normal H_2L_2 molecule (Franklin, 1964). In all cases, the heavy chain is incomplete. In most cases, the cells producing the abnormal heavy chain do not produce light chains of any type (Franklin & Frangione, 1976).

Biosynthetic and structural studies have suggested that the short heavy chains are primary gene products which result from variably sized internal deletions. The present studies were initiated to provide a system in which more detailed biochemical experiments could be performed.

The serum of patient OMM contained an apparently electrophoretically homogeneous protein of γ mobility, but was found, on more detailed analysis, to contain two distinct proteins. The major protein had a molecular weight of approximately 80–85,000 daltons and displayed γ_3 heavy chain antigenic determinants, but did not bear detectable κ or λ light chain antigens. The minor component appeared to be an intact $(\gamma_3)_2\lambda_2$ protein (Adlersberg *et al.*, 1978). Detailed structural analysis of the smaller protein indicated that it had an amino terminal glycine residue followed by a region of identical sequence to that of the normal γ_3 hinge region. Although the protein appeared to contain an amino terminal deletion, the lack of a normal amino terminus could have been the result of either intra- or extracellular proteolytic digestion (Adlersberg, Franklin & Frangione, 1975).

MATERIALS AND METHODS

Bone marrow cells were obtained at the time of diagnostic marrow aspiration. The cells were drawn into a heparinized syringe. They were added to 15 ml of heparinized (0·075 mg/ml) Eagle's minimal essential medium (MEM) which did not contain valine, threonine or leucine (−VTL medium). The cells were centrifuged at 4°C at 1000 rev/min for 8 min in an

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International PR-J centrifuge. The supernatant medium was discarded, the cells were washed twice more, and the buffy coat resuspended in 5 ml of medium which contained 1/20 of the normal concentration of valine, threonine and leucine of MEM (1/20 VTL medium). The cells were counted and the concentration adjusted to $2{\cdot}5\times 10^6$ cells per ml. 20 μCi each of ^{14}C-labelled valine, threonine and leucine were added. Aliquots of cells were removed at 10 min, 20 min, 30 min, 60 min, 4 hr and 6 hr, and placed in an ice bath. Iodoacetamide was added to a final concentration of 0·2 M. The cells were centrifuged (1000 rev/min, 10 min, 4°C). The supernatant medium containing the labelled secretions was removed and iced for further analysis. The cells were washed in 10 ml ice cold medium twice. They were then suspended in 9 ml of cold distilled water for 1 min, after which 1 ml of ten-fold concentrated Earle's balanced salt solution was added to render the cell suspension isotonic. This lysed the residual marrow erythrocytes. The remaining cells were washed once more, then lysed in 0·5% nonidet P-40 (NP-40) and spun at 100,000 *g* (Borun, Scharff & Robbins, 1967). The aliquots of the 100,000 *g* supernatant and the secretions were then analysed by sodium dodecylsulphate (SDS) acrylamide gel electrophoresis. Additional aliquots were immunologically precipitated with antisera specific for either immunoglobulin heavy or light chains. Precipitations were done by the direct technique, using a 1:50 dilution of normal human serum as a carrier in previously ascertained two-fold antibody excess. The precipitates were washed extensively, dissolved in 2% SDS, placed in a boiling water bath and then electrophoresed as previously described (Zolla *et al.*, 1970). Slab gels were run in a Tris–glycine SDS buffer system (Laemmli, 1970).

A long term cell line was established from the peripheral blood of the patient (OMM_{cc}). 50 ml of peripheral blood were drawn into a heparinized syringe. The white blood cell count at this time was 4100/mm^3, with 88% of the cells being lymphocytes. The anti-coagulated blood was allowed to settle for 30 min at 37°C. The leucocyte-rich plasma was removed and centrifuged at 1000 rev/min for 5 min in an International table-top centrifuge. The pellet was suspended in RPMI 1640 containing 20% foetal calf serum, 4–8 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at a concentration of 5×10^6 cells per ml in Falcon flasks (75 cm^2). The cells were maintained in a 5% CO_2 in air atmosphere and were inspected daily. Half the volume was replaced once a week with an additional half volume added 3–4 days later. An attempt was made to maintain the viable cell concentration between 10^6 and 5×10^6 cells per ml. After 2 weeks in culture, the number of viable cells decreased perceptibly. At approximately 4 weeks of culture, clumps of viable cells were clearly visible. By 6–8 weeks of culture, the cell clumps were the major elements in the culture. The cells then required addition of fresh medium 2 to 3 times weekly.

Generation time experiments were performed by resuspending the cells at various concentrations in fresh medium. The cells were allowed to grow and were counted every 24 hr over 5 days.

The cells could be cloned in soft agar using a feeder layer made up of cells obtained from the rat embryo cell line 1706 (provided by Dr M. D. Scharff) (Coffino, Laskov & Scharff, 1970). The 1706 cells were trypsinized and allowed to form monolayers in 60 mm tissue culture dishes. 3·0 ml of a solution of 0·22% agarose in complete tissue culture medium was pipetted into each dish and allowed to set. The cultured cells were suspended in the agarose plating medium at a final concentration of 1000 cells per ml. 1·0 ml of the cell–agarose suspension was layered on top of the base layer. The clones became apparent after 7 to 10 days and could be picked from the agar after 2–3 weeks and grown to mass culture.

Karyotypic analysis by quinacrine staining and Giemsa staining after trypsin treatment was performed by Dr K. Hirschorn of the Department of Pediatrics of the Mt Sinai Medical School.

RESULTS

When OMM bone marrow cells were incubated with radioactive amino acids in short term tissue culture, the incorporation into total cell protein and immunoglobulin was linear for 6 hr. Secretion of labelled protein was not detectable for 60 min; however, after the initial lag, it too was linear for the 6 hr duration of the experiment. Two major molecular species bearing γ antigenic determinants were synthesized and secreted during the experiment. Fig. 1 shows the electropherogram of radioactive immunologically precipitated cytoplasm and secreted material. The 6 hr secretions contained two distinct protein peaks (Fig. 1a). The major protein had an estimated molecular weight of 89,000 daltons and was precipitable with anti-γ antiserum. It appeared to represent a dimer of the HCD fragment. The second peak had a broader electrophoretic spectrum which overlapped the murine $\gamma_{2b}\kappa_2$ marker. It was precipitable with both anti-Fc and anti-Fab antiserum and had an estimated molecular weight, in this gel system, of 180,000 daltons and probably represented the intact H_2L_2 molecule.

The 6 hr cytoplasm, prepared by precipitation with anti-γ antiserum, contained a major protein with an estimated molecular weight of 87,000 daltons. It was not precipitable with an antibody which reacted with both κ and λ light chains, and hence had no light chain antigenic determinants (Fig. 1b).

When the anti-γ immunological precipitates of the secreted material were reduced, alkylated and electrophoresed on 7·5% acrylamide gels, two peaks were seen with molecular weights of 69,000 and 50,000 daltons, respectively. Gels of the reduced and alkylated anti-Fab precipitates revealed the 69,000

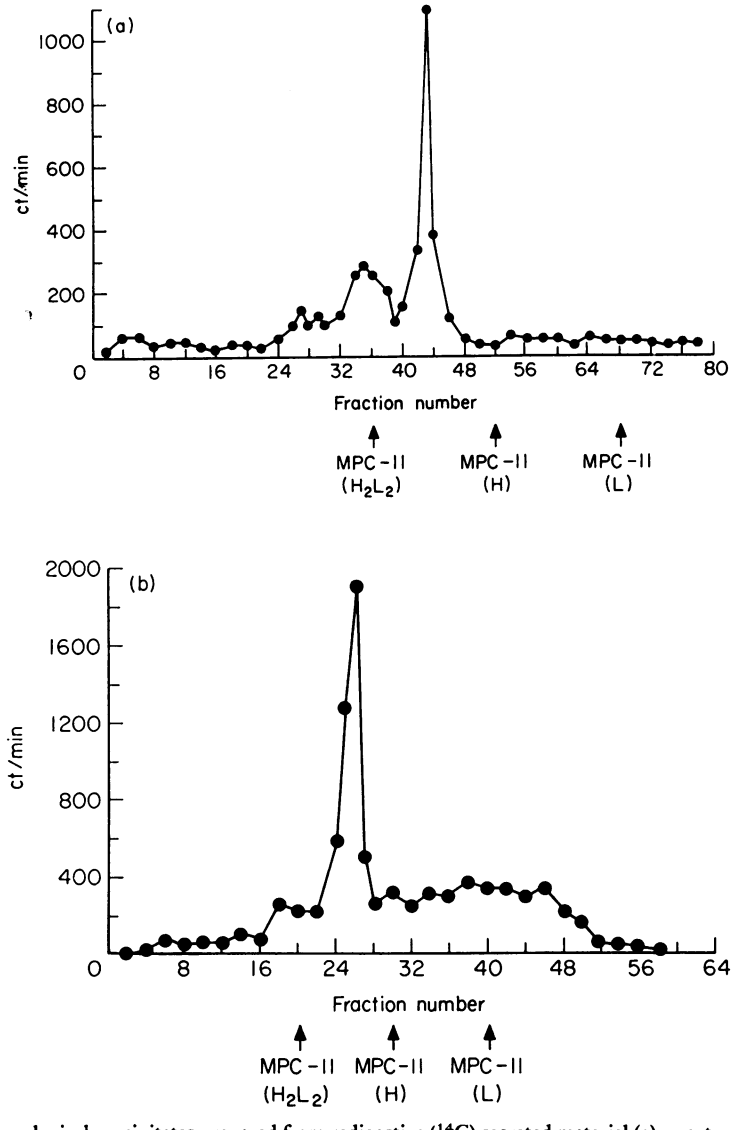

FIG. 1. Immunological precipitates prepared from radioactive (^{14}C) secreted material (a) or cytoplasm (b) with antibody directed against Fcγ were dissolved in 2% SDS in 0·01 M phosphate buffer and heated in a boiling water bath for 1 min. They were electrophoresed on 20 cm cylindrical 5% polyacrylamide gels containing 0·1% SDS and 0·01 M phosphate. Each sample was mixed with ^{3}H-labelled secretions from the murine IgG 2b-producing myeloma MPC-11. The marker contained H_2L_2 (150,000 daltons), H (55,000 daltons) and L (23,500 daltons) chains. The positions of the markers are indicated by arrows.

dalton peak. The larger molecule probably represented the normal γ_3 H chain, while the smaller was the deleted molecule. The cytoplasm was precipitated with anti-γ antisera (Fig. 1b), then reduced, alkylated and electrophoresed. A single peak with an approximate molecular weight of 45,000 daltons was noted (Fig. 2). Light chain peaks were small in both reductions, presumably because of the low incorporation and loss in the background of the co-migrating heavily-labelled normal L-chain marker.

Serial samples, taken at times from 10 to 360 min of incubation, gave no evidence of degradation of a normal-size heavy chain. At 10 min after addition of radioactive amino acids, the major form of the OMM protein was the 85,000 dalton disulphide-linked dimer, suggesting that, within the cell, dimerization took place very rapidly.

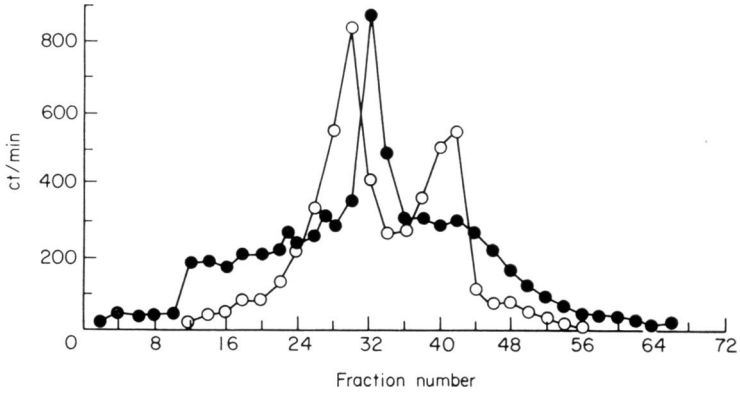

FIG. 2. An anti-Fcγ cytoplasmic immune precipitate was prepared, dissolved in 2% SDS and mixed with 0·15 M 2-mercaptoethanol. After 30 min at 37°C, 0·2 M iodoacetamide was added for 1 hr. The sample was then dialysed against running buffer containing 0·015 M mercaptoethanol. Electrophoresis was carried out on 7·5% SDS containing acrylamide gels. The electropherogram of the reduced and alkylated OMM protein is represented by (●), and the co-electrophoresed MPC-11 heavy and light chains are indicated by (○).

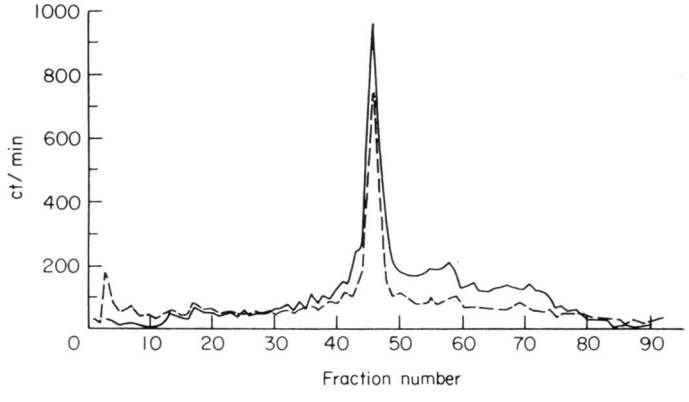

FIG. 3. Electrophoresis of immunologically precipitated (anti-γ) ^{14}C-labelled amino acid-labelled 4 hr secretions (— — —) and ^{3}H-labelled 3·5 min cytoplasm (———) obtained from the OMM cultured cells. The tritiated and ^{14}C-labelled material were precipitated, mixed and gelled.

Proteins bearing immunoglobulin antigenic determinants comprised approximately 15% of the total cytoplasmic radioactivity up to 30 min. Between 30 and 60 min, the proportion dropped to 8% and remained at that level throughout the remainder of the experiment. At 360 min, approximately 40% of the total extracellular trichloracetic acid-precipitable radioactivity was found in immunoglobulin.

When the cultured cells were studied by similar labelling techniques, only a single major immunoglobulin molecule was noted in the cytoplasm and secreted material. The protein secreted after 4 hr was identical in antigenicity and electrophoretic mobility to that found intracellularly after 3·5 min of labelling (Fig. 3).

Pulse chase experiments performed with the cultured cells revealed that at all times, from 3·5 min to 3 hr after addition of label, the major intracellular immunoglobulin was the dimer of the short heavy chain.

The tritiated cellular and secreted immunoglobulins were reduced and alkylated and co-electrophoresed on the same gel with reduced and ^{14}C-labelled carboxymethylated OMM serum protein (provided by Drs J. Adlersberg and E. C. Franklin). The mobility of the reduced and alkylated short serum protein was identical to that found in the cytoplasm and secreted material produced by the cultured cells. The intact γ_3 heavy chain isolated from serum, OMM_L, did not correspond to any of the immunologically precipitable proteins found in either the cytoplasm or secretions (Fig. 4).

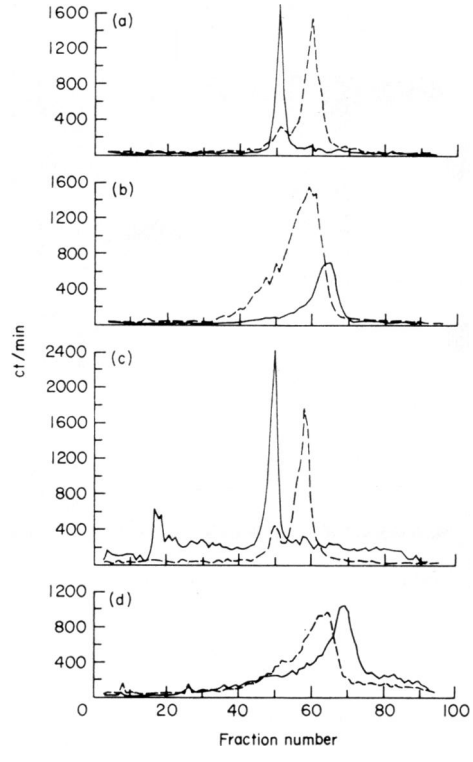

FIG. 4. The intact OMM_L heavy chain was reduced and carboxymethylated and mixed with immunologically precipitated secretions (———) (a), reduced and alkylated precipitated secretions (———) (b), precipitated cytoplasm (———) (c) and precipitated reduced and alkylated cytoplasm (———) (d). Electrophoresis was carried out in 5% acrylamide gels as above. None of the cultured cells associated peaks corresponded to OMM_L (— — —) either before or after reduction.

The OMM cells were cloned in soft agar. Initially, cloning efficiency was very low (0·1%). After the cells had been in culture for 18 months, the cloning efficiency increased to between 5 and 10%. Several clones were isolated and analysed. Fig. 5 shows a comparison of the labelled secretions from the parent culture, a culture which had been frozen in liquid nitrogen for 1 year, and OMM clone 1. All appear to be identical.

Karyotypic analysis of the parent culture revealed a normal pattern. Quinacrine fluorescence (Fig. 6) revealed a banding pattern identical to that seen in normal human cells. Similar results were obtained by the trypsin–Giemsa technique.

Electron microscopy of the cultured cells (kindly performed by Dr D. Zucker-Franklin) revealed a lymphoblastoid appearance, with the cytoplasm containing primarily free polyribosomes. Substantial numbers of Herpes-type viral particles were noted. A small amount of rough endoplasmic reticulum could be seen in most cells.

When the cultured cells were incubated in petri dishes in fresh medium at various concentrations, they had a doubling time of approximately 30 hr. The maximum achievable viable cell concentration ranged between 1·2 and 2×10^6 cells per ml. Although higher cell densities could be achieved, the percentage viability was then diminished.

The cells were originally grown in RPMI 1640 supplemented with 20% foetal calf serum. With increasing time in culture, it was possible to reduce the foetal calf serum concentration to 5% without altering the growth characteristics of the cells. The cells could also be grown in γ-globulin-free foetal calf serum, allowing concentration and immunochemical analysis of the secreted proteins. The HCD protein

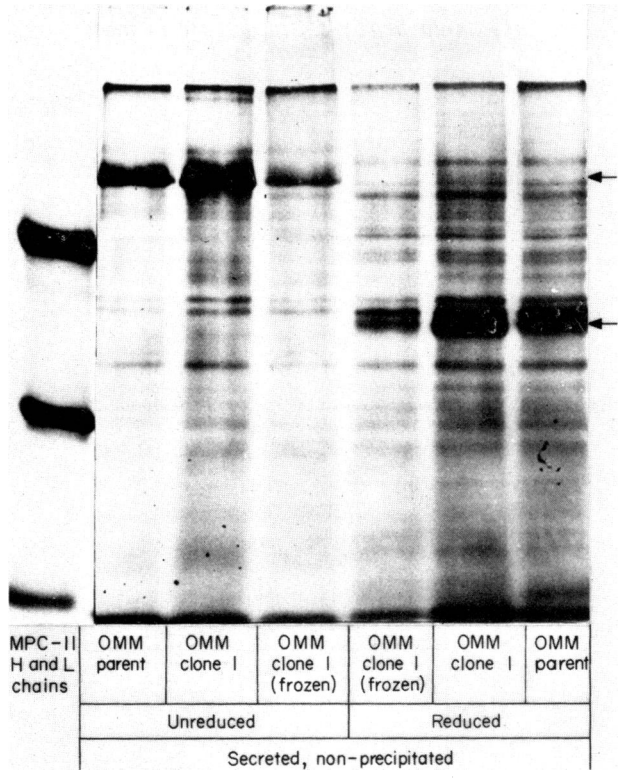

FIG. 5. Three months after the OMM line was established in culture, several vials of cells, designated OMM parent, were frozen in 15% DMSO under liquid N_2. A cloned subline derived from the parent was kept in continuous culture (OMM clone 1) and was also stored frozen for 1 year before being placed in culture again (OMM clone 1, frozen). Cells from each of the two cloned OMM populations and OMM parent were labelled with radioactive amino acid for 4 hr. The cells were then removed and the supernatant medium containing the secreted OMM protein was adjusted to 2% SDS. Aliquots of the supernatant were electrophoresed on a Tris–glycine SDS–polyacrylamide slab gel (Laemmli, 1970). Channel 1 shows the MPC-11, H and L chains. Channels 2, 3 and 4 show the unreduced secretions, and 5, 6 and 7 the secretions after reduction and alkylation. The OMM protein is indicated by the arrow.

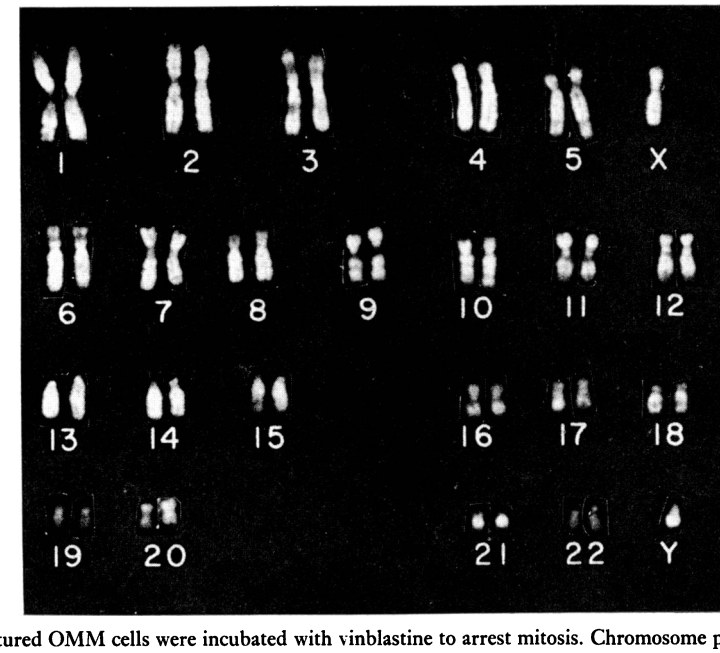

FIG. 6. Cultured OMM cells were incubated with vinblastine to arrest mitosis. Chromosome preparation and quinacrine staining were performed according to the method of Caspersson *et al.* in the laboratory of Dr. K. Hirschorn, Mt Sinai Medical School, New York.

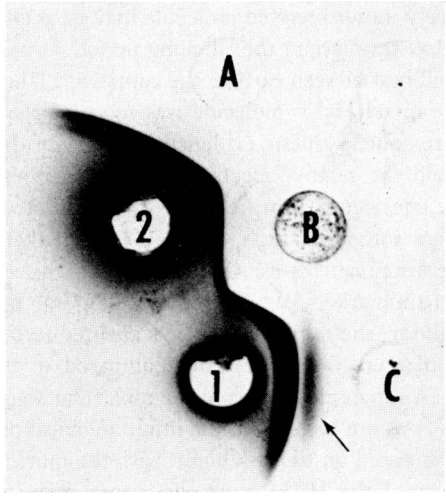

FIG. 7. Wells (A) and (C) contain the HCD protein isolated from the serum of patient OMM. Well (B) contains the HCD protein isolated from the concentrated spent tissue culture medium bathing the OMM cells. Well 1 contains antibody to the heavy chain of the intact M protein (OMM_L) found in patient OMM's serum. Well 2 contains antibody produced by a rabbit immunized with the defective serum protein. The second band in well (C) (arrow) is due to contamination of the short serum protein with some of the intact OMM_L. The reactions of immunological identity between the defective serum protein and the protein secreted by the cells in tissue culture are apparent with both antisera.

secreted by the cultured cells was electrophoretically and immunologically identical to the short protein isolated from the serum (Fig. 7). However, preliminary amino acid sequence analysis of the protein isolated from the spent tissue culture medium indicated that it differed from the serum protein by having an N-terminal pyrrolidone carboxylic acid.

DISCUSSION

Heavy chain disease cells are characterized by two defects in γ-globulin synthesis. Most synthesize a defective heavy chain which contains an internal deletion. The cells from most patients also fail to produce any detectable light chain.

The serum of patient OMM contained two proteins bearing γ_3 antigenic determinants. Analysis of these proteins suggested that the deleted protein was identical to the heavy chain of the intact H_2L_2 myeloma protein from residue 222 to the carboxy terminus (Adlersberg *et al.*, 1975, 1978). The deleted molecule was unusual for an HCD protein, since the short chain did not contain the normal amino terminus. Its absence suggested that the OMM protein may have been a primary degradation product.

The short term tissue culture experiments were similar to those performed in other HCD patients (Ein, Buell & Fahey, 1969; Buxbaum, Franklin & Scharff, 1970; Usui *et al.*, 1971; Buxbaum & Preud'homme, 1972). There was no indication of the generation of the smaller protein from a normal IgG or γ heavy chain precursor. A 7S molecule was present, but the relationship between it and the dimer of the heavy chain fragment could not be established by these experiments. It seems likely, however, that the H_2L_2 molecule synthesized by the marrow cells in short term culture was the intact γ_3 monoclonal protein. The fact that the unreduced molecule migrated more slowly on the gels, i.e. had an apparent molecular weight greater than the murine IgG 2b marker, and that the reduced and alkylated heavy chain migrated behind the murine heavy chain marker, supports this contention, since normal human γ_3 heavy chain is larger than the MPC-11 γ chain.

The H_2L_2 molecule was seen only in the long-labelled secretions of the marrow cells. It was not seen in the cytoplasm. A quantitatively minor secreted molecule may be preferentially seen in the secretions, because the secretions accumulate throughout the labelling period. It will comprise a smaller fraction of the cytoplasmic proteins and will best be seen early in the course of a labelling experiment. Alternatively, one could hypothesize that the intact H_2L_2 molecule was more efficiently secreted than the defective HCD fragment. However, there is little kinetic evidence in favour of this hypothesis.

Although the antigenicity and the relative electrophoretic mobilities of the reduced and alkylated immunological precipitates are consistent with the intact γ_3 and deleted γ_3 heavy chain molecules, the calculated molecular weights are somewhat high, i.e. 69,000 *vs* 58,000 and 50,000 *vs* 40,000 daltons, respectively. We feel that these discrepancies are the result of local overloading of the gels in the regions of the normal heavy and light chain markers. When the murine IgG 2b marker is reduced in the presence of large amounts of rabbit antibody, the rabbit antibody is also reduced, overloading the gel for normal heavy and light chains. The markers then run faster compared to the proteins migrating in other regions of the gel, resulting in an apparent increase in the molecular weights of the non-marker proteins (Shapiro & Maizel, 1969). This was not seen when the immunological precipitates of the cytoplasm and secreted material were electrophoresed on SDS slab gels with the murine markers in adjacent channels. The monomer and dimer forms of the HCD fragment always migrated in positions consistent with molecular weights of 39,000–40,000 and 80,000–85,000 daltons, respectively.

The limited duration of the marrow studies did not allow us to eliminate the possibility of degradation of nascent chains. The availability of the long term line allowed further exploration of this possibility. The fact that the line and its subclones synthesized only the HCD protein established that the aberrant protein was a clonal product. However, the early structural analysis of the protein secreted by the cells suggested that the aberrant γ_3 chain produced by the clone had undergone a small amount of N-terminal proteolysis in the patient's serum. It was probable that the OMM cell line which became adapted to culture was a variant of the patient's original neoplastic cell line which synthesized the intact γ_3 H_2L_2 protein.

There is good evidence that immunoglobulin heavy and light chains are coded by genes on separate chromosomes (Dreyer, Gray & Hood, 1967). It is not likely that the defective heavy chain and the lack of light chain production were the effect of abnormalities in two structural genes, although a rearrangement affecting the chromosomes carrying the information for both chains was a possibility. However, even a small translocation involving two chromosomes should be detectable by current banding techniques. The presence of a normal karyotype and banding pattern strengthens the contention that a major chromosomal event had not occurred.

These studies support the hypothesis that the OMM HCD protein is a clonal product resulting from a defect in a structural gene. The precise nature of the gene defect and the abnormality resulting in the absence of light chain synthesis have not yet been elucidated. The availability of the OMM cell line has allowed the isolation of the mRNA coding for the aberrant protein, analysis of the primary translation product, and some tentative judgements about the nature of the primary gene defect (Buxbaum & Alexander, 1977; Alexander, Barritault & Buxbaum, in preparation).

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