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**Abstract**

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# FURTHER STUDIES ON AN ELEVENTH CASE OF HEAVY ($H\gamma 1$) CHAIN DISEASE — BIOSYNTHETIC STUDIES —

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*Abstract*: *In vitro* quantitative biosynthetic studies were carried out on bone marrow cells obtained from an eleventh case with gamma heavy chain disease. The findings indicate that neither cytoplasmic nor extracellular degradation was responsible for the presence of the gamma heavy chain fragment in serum. The absence of a covalent-bound light chain was also confirmed.

The first case report of gamma heavy chain disease showed that extracellular catabolism appears not to be responsible for the existence of anomalous protein in patient serum (1). Several biosynthetic studies of the protein were carried out with peripheral blood lymphocytes and with bone marrow cells which showed the probable source of the labelled protein (2-4).

*In vitro* quantitative labeling studies carried out with cells obtained from a patient with gamma heavy chain disease indicated that neither of the incomplete chains appeared to be products of a primary intra- or extracellular degradation process (5). The present studies with an eleventh case of the disease is consistent with these findings.

## METHODS

For biosynthetic studies of the Fc fragment, bone marrow cells were aspirated in heparinized syringes from the iliac crest of the patient. The aspirates were transferred to 10 ml of Eagle's minimal essential medium (MEM) with 1/20 the normal concentration of valine, threonine, and leucine, and 100 units of heparin (1/20 medium) (6, 7). The marrow cells were washed twice with 1/20 medium and then suspended in distilled water for 60 seconds to lyse the red cells. The cell suspensions were then brought to isotonicity with 2×MEM.

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Cells were washed twice with 1/20 medium and resuspended at a final concentration of $5\times10^6$ to $1\times10^7$ cells/milliliter. These procedures were carried out at 4°C. 20 $\mu$ Ci each of $^{14}$C-labeled valine, threonine, and leucine was then added to the cell suspensions for continuous labeling experiments. After exposure for 60 seconds to a gentle stream of air containing 5% $CO_2$, the incubation was started in a shaking water both at 37°C. Equal aliquots were then removed at a given time and placed in an ice bath to stop protein synthesis.

For the prepartion of labeled cytoplasmic material, cells after incubation were washed twice with cold MEM, treated with distilled water to lyse red cells, and washed twice with cold MEM. Finally cells were suspended in phosphate buffered saline and disrupted by repeating freezing and thawing, three times. Nonidet P-40 was then added to the solution at a final concentration of 0.5%, and it was spun at 100,000 g for 20 minutes to remove nuclei and ribosomes (6). To quantitate each immunoglobulin and molecular subunits of IgG produced, they were precipitated with specific antisera obtained commercially (Behringwerke AG, Marburg, Lahn, Germany and Handai Biken, Suita, Osaka, Japan). Antiserum (100 $\mu$l) was added to the cytoplasm or secreted material in the presence of an appropriate volume of "carrier" normal human serum (2-20 $\mu$l). Preliminary precipitin curves were carried out to ensure that all precipitations were performed in antibody excess. The precipitates were washed five times with cold phosphate buffered saline, and subjected to counting of radioactivities. Total macromolecules synthesized were determined by precipitation with 5% trichloroacetic acid in the presence of 5 $\mu$l of normal human serum. Radioactivities in precipitates formed by both methods were analysed after their dissolving in 1% sodium dodecyl sulfate (SDS) at 37°C for 60 minutes.

For the identification of biosynthetic products, the cytoplasm and secreted material were subjected to polyacrylamide electrophoresis (8) directly or after precipitation with specific anti-sera. The products of synthesis were identified by comparing their mobility to known marker proteins synthesized by bone marrow cells of human IgG myelomas. The washed precipitates were dissolved in 2% SDS with 0.03 M iodoacetamide and 0.01 M sodium phosphate buffer, pH 6.7, and placed in a boiling water bath for 2 minutes (8). The SDS-dissociated samples were subjected to electrophoresis on 12 cm, 9% acrylamide gels containing 0.1% SDS according to MAIZEL'S SDS-phos gel electrophoresis (8). In this gel system, IgG, Fc fragment, and light chain were separated appropriately. For fractionation of gels, a Maizels gel grinder (Savant Instruments, Inc., Hicksville, N. Y.) and a gel slicer (Osaka Shoko, Inc., Toyonaka, Osaka) were employed. The ground gel fractions were counted directly in a 10 ml of Bray's scintillation cocktail. The sliced gels were suspended in 0.7 ml of 90% NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Illinois), heated at 50°C for 2 hours, and counted in the same cocktail. Counting of radioactivity was performed in an Aloka LSC-502 or an Aloka LSC-601B liquid Scintillation counter.

## RESULTS

Seventy-two per cent of radioactivity incorporated in cytoplasmic materials was solubilized by freezing-thawing-Nonidet P-40 treatment and centrifugation at 60,000g. After four hours incubation, 43,000 cpm and 94,000 cpm of radioactivity were incorporated in secreted materials and in cytoplasmic materials, respectively. As shown in Table 1, the radioactivity in

TABLE 1 INCORPORATION OF $C^{14}$-LABELED AMINO ACIDS BY BONE MARROW CELLS OF A HEAVY ($H\gamma^1$) CHAIN DISEASE.

| Protein fraction precipitated by specific anti serum | Radioactivity in secreted materials | Radioactivity in cytoplasmic materials |
|---|---|---|
| IgG | 39,800 cpm | 41,100 cpm |
| IgA | 1,700 | 1,100 |
| IgM | 3,100 | 2,900 |
| K | 1,900 | 1,400 |
| L | 2,100 | 1,800 |
| Fab | 1,300 | 1,300 |
| Fc | 41,000 | 38,200 |
| Alb | 2,700 | 1,200 |
| 5% TCA | 43,000 | 94,000 |

To quantitate each immunoglobulins and molecular subunits of IgG produced, they were precipitated by specific anti sera. Total biosynthetic product was precipitated by 5% trichloroacetic acid (TCA). Note $C^{14}$-radioactivity was incorporated only in Fc and IgG fractions, but not in Fab fraction suggesting synthesis of only Fc fragment. For a control, the radioactivity precipitated by anti-albumin serum is also presented.

both secreted materials and cytoplamic materials was precipitated by anti-IgG or anti Fc-serum, but not with anti-IgA, anti-IgM, anti-K, anti-L, anti-Fab or anti-albumin. Ninety-five and three-tenths per cent and 43.7% of radioactivity in secreted materials and in cytoplasmic materials were precipitated by anti-Fc and anti-IgG, respectively. These results suggested that most secreted materials and half of cytoplasmic materials synthesized in four hour incubations were Fc fragment. Neither light chains nor Fd portion of IgG molecule seemed to be present in either preparation. This conclusion was confirmed by time course studies of bone marrow incubation (Fig. 1). Radioactivity in secreted materials was accummulated after a 60 minutes lag and most radioactivity was precipitated with anti-IgG and anti-Fc sera but not with anti-Fab serum. The radioactive cytoplasmic materials increased in proportion to incubation time, and the concentration of radioactive materials precipitated by anti-IgG or anti-Fc was maintained in the same range after five hours of incubation. No material precipitated with anti-Fab could

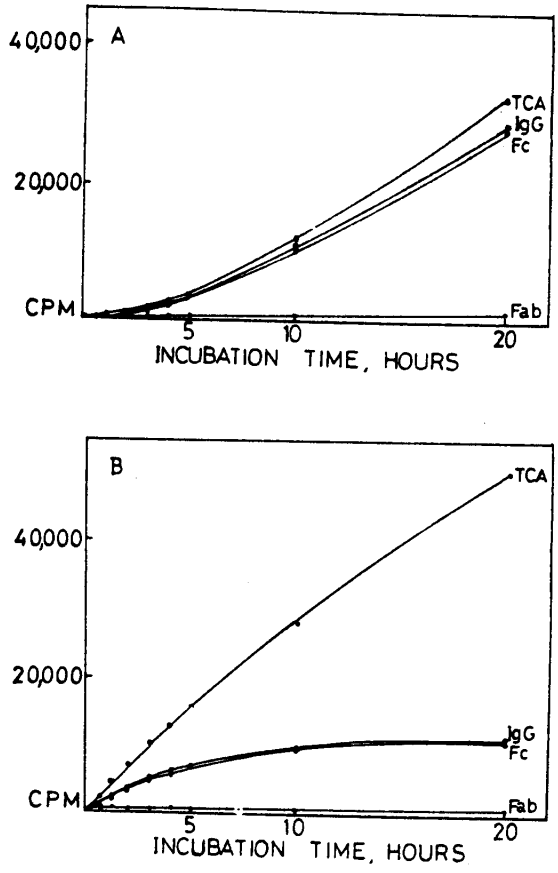


Fig. 1. Time course studies on biosynthesis of M-component by bone marrow cells.

A. Radioactivities in secreted material. B. Radioactivities in cytoplasmic material. Immunoglobulins and molecular subunits of IgG were precipitated by specific anti-sera. TCA, precipitated by 5% TCA; Fc, precipitated by anti-Fc; IgG, precipitated by anti-IgG; Fab, precipitated by anti-Fab. Anti-K, and anti-L gave the same results as anti-Fab. Note radioactivities were precipitated only by anti-Fc or by anti-IgG in both preparations.

be found in the cytoplasm.

Secreted materials and cytoplasmic materials were electrophoresed in acrylamide gels before and after the precipitation with anti-Fc and anti-IgG. The patterns of fractionation are shown in Figs. 2 and 3. Two main peaks appeared in both secreted and cytoplasmic materials, and only one peak corresponding to Fc-fragment was found after immunological precipitation.

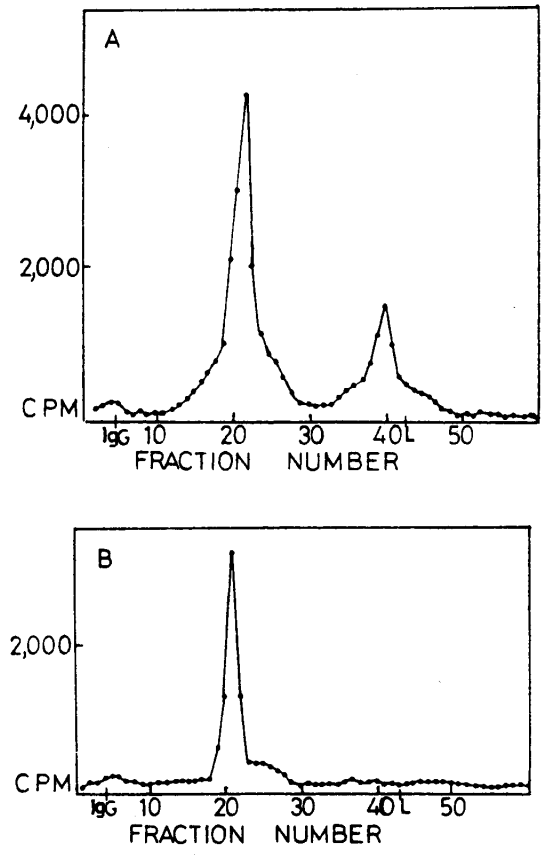


Fig. 2. Fractionation in SDS-phos gel electrophoresis of $^{14}C$-labeled secreted material of bone marrow cells.

The supernatant of culture medium was electrophoresed. A. Without immunologic precipitation, B. After precipitation with anti-Fc or anti-IgG serum. Each sample was run with $^{3}H$-labeled IgG and light chain (L) synthesized by bone marrow cells of K type human IgG myeloma patient. The anode is at the right and migration is toward the right. The migration of these markers is indicated on the abcissa.

## DISCUSSION

Radioimmunoelectrophoretic and immunofluorescent studies on bone marrow cells and lymph node cells suggested that cells synthesizing the abnormal protein were present in these preparations, and these cells did not seem to produce an immunoglobulin light chains (2-4).

Bone marrow cells from our case (Yok) were incubated with radioactive amino acids in a short-term tissue culture. Samples of soluble cytoplasmic

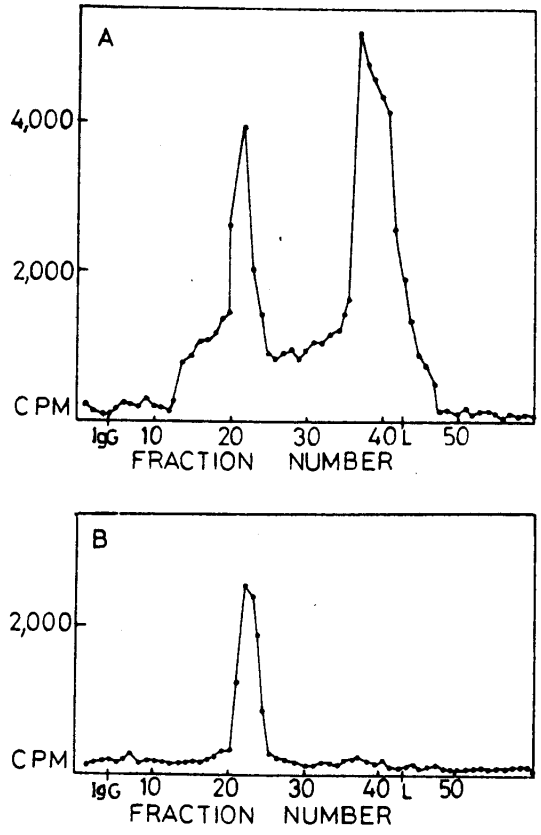


Fig. 3. Fractionation on SDS-phos gel electrophoresis of $^{14}C$-labeled cytoplasmic material of bone marrow cell.

The cytoplasmic material was electrophoresed. A. Without immunologic precipitation, B. After precipitation with anti Fc or anti-IgG serum. They were electrophoresed with labeled human immunoglobulin components as markers. The migration of these markers is indicated on the abscissa.

extracts and secreted materials were examined by immunologic precipitation with specific anti-sera, and acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. These methods are thought to be more sensitive and quantitative than radioimmunoelectrophoresis and immunofluorescence (9). Time course studies on the marrow cells were also performed. By these experiments, it was found that bone marrow cells do not synthesize neither whole IgG molecules nor the whole heavy gamma chain. Light chains could not be found in both preparations while cells produced only the anomalous protein related to Fc portion of IgG.

In the course of our studies, immunofluorescent and *in vitro* labeling

studies on cells obtained from a patient with gamma heavy chain disease (Va) have been reported from other laboratories (5). The report showed no evidence of post-ribosomal intracellular degradation of normal size heavy chain in the case, and intracellular polymerization of the aberrant peptides appeared to take place in a similar manner to cells synthesizing intact heavy chains. These results are compatible with our own observations.

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