In vitro Incorporation of C^{14}-Lysine into Bence-Jones Protein.* (27438)

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It has been generally assumed that the neoplastic plasma cells in the bone marrow of persons afflicted with multiple myeloma are the source of Bence-Jones protein(1,2,3,4,5) although no direct experimental evidence for this view has been obtained.

The present experiments were performed to determine whether an *in vitro* culture of bone marrow cells from a myeloma patient excreting Bence-Jones protein was capable of producing Bence-Jones protein. By the use of C^{14}-lysine it has been demonstrated that cells derived from such a patient not only formed a fraction having the properties of Bence-Jones protein, but that this fraction accounted for practically all of the radioactivity present in the non-dialyzable portion of the cell culture fluid.

Experimental. In vitro culture of bone marrow cells. Approximately 5 ml of freshly aspirated bone marrow from a middle-aged adult female myeloma patient,‡ excreting large amounts of urinary Bence-Jones protein, were mixed under sterile conditions with 50 ml of unlabeled nutrient medium (a modified Eagle's medium mixed with an equal amount of human serum)(6). The mixture was centrifuged for 3 minutes and the pellet of cells then resuspended in 12 ml of fresh nutrient medium containing C^{14}-lysine. Five ml of this cell suspension were placed in the upper chamber of 2 specially designed incubation flasks(6) and the same labeled nutrient medium was added to the lower reservoir compartments. The suspensions of cells were gently shaken at 37°C and aerated with a mixture of 95% O_2-5% CO_2 for periods of 72 hours and 168 hours. At the end of each period the cell suspensions were centrifuged and the cells examined histologically. It was seen that the cells were intact after 72 hours of incubation but had disintegrated after 168 hours.

Detection of radioactivity in the non-dialyzable fraction of the culture fluid. After incubation, both the cell-free culture fluids and an aliquot of the labeled nutrient medium were rigorously dialyzed, first against running tap water, for 2 days, then against 3 changes of physiological saline containing 30 mg % unlabeled lysine, and finally against 6 changes of physiological saline alone.

Aliquots of dialyzed materials were deposited on planchets, air-dried to layers of "infinite" thickness, and radioactivity measured in a Geiger-Müller gas flow chamber.

Isolation of Bence-Jones protein. Three liters of urine obtained from the patient were adjusted to pH 5.2 with acetic acid and dialyzed twice against 3M $(NH_4)_2SO_4$. The dialyzed material was centrifuged, the precipitate dissolved in distilled water, then dialyzed successively against tap water, distilled water, and physiological saline until free of sulfate ion. The material thus obtained precipitated at 58°C and redissolved upon boiling, which is characteristic of Bence-Jones protein. A portion of the isolated protein was dialyzed against Veronal buffer; it showed upon centrifugation in the Spinco Analytical Ultracentrifuge (Model E) the sedimentation characteristics typical of Bence-Jones protein.

Fractionation and characterization of dialyzed culture fluid by starch zone electropho-

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‡ The patient was a 58-year-old female, first admitted to the Outpatient Department on 5/5/55. X-ray examination established the presence of extensive osteolytic lesions in all of the visualized portions of the skeleton. Clinical laboratory examination of urine revealed a 2 plus albuminuria, due to presence of Bence-Jones protein. Examination of plasma proteins showed normal values for non-protein nitrogen, albumin and globulin. Histological examination of a small piece of aspirated sternal bone marrow revealed a marked plasmocytosis, including many immature forms. erythroid and myelogenous cells scattered throughout. The diagnosis made was multiple myeloma.

resis. The starch bed of a Reco starch zone electrophoresis apparatus (Model E-800-2) was separated lengthwise by a Lucite divider into 2 unequal compartments, the smaller approximately 1 cm and the larger approximately 19 cm in width. These 2 compartments were filled with a paste consisting of potato starch mixed with a suitable quantity of Veronal buffer pH 8.6, ionic strength, 0.1. A control mixture consisting of Bence-Jones protein (isolated from the patient's own urine) and nutrient medium, after dialysis against Veronal buffer, was introduced into the smaller compartment. The larger compartment received the remainder of the previously dialyzed culture fluid after a further dialysis against Veronal buffer. Electrophoresis was carried out for 68 hours at 250 V. and 20 ma. The starch beds were sectioned crosswise at 1-cm intervals into 28 fractions. The fractions were extracted with equal amounts of Veronal buffer, and the protein content of an aliquot of each was determined by the Lowry method(7). A second aliquot of each extract was placed on planchets and the radioactivity measured.

Characterization of radioactive starch zone fractions. Pooled extracts of the most radioactive fractions (No. 23, 24, 25) corresponding to the positions of the Bence-Jones protein and gamma globulins (Fig. 1 and 2) were dialyzed against physiological saline and subjected to further characterization and fractionation as follows:

a) Heat fractionation. 15 mg of urinary Bence-Jones protein isolated from the patient's own urine and 15 mg of human gamma globulins (Poliomyelitis Immune Globulin, Lederle) were added to an aliquot of the pooled radioactive extract as carriers. The mixture in saline was heated at 58°C for 4 min., and the supernatant from the resultant precipitated mixture decanted after centrifugation. The supernatant was subjected to 2 additional cycles of heating and centrifugation, after which no further precipitate was obtained.§ The pooled precipitates were washed once with saline, twice with distilled water, acidified with 1N acetic acid and boiled. The material (Bence-Jones protein) which dissolved completely by this procedure was placed on planchets and its radioactivity determined.

The final supernatant was acidified with acetic acid and boiled. The resulting precipitate (gamma globulins) was removed by centrifugation, washed 3 times with distilled water, dissolved in 0.02N NaOH, and its radioactivity measured as was that of the supernatant remaining after boiling.

b) Fractionation by immunological reaction. To a second aliquot of the pooled extract were added 7 mg of human gamma globulins and 28.1 mg of urinary Bence-Jones protein (isolated from the patient's own urine) as carriers together with an appropriate amount of purified rabbit anti-human gamma globulins. The mixture was incubated at 37°C for 30 minutes and then at 3°C for 24 hours.‖ The resulting precipitate was removed by centrifugation, washed twice with saline and once with distilled water. The protein in the supernatant was precipitated with trichloroacetic acid at 100°C and washed 3 times with distilled water. Both precipitates were then resuspended in 0.02N NaOH and their radioactivity measured.

c) Fractionation by differential centrifugation. To a third aliquot of the pooled extract, 24 mg each of urinary Bence-Jones protein and human gamma globulins were added as carrier. The mixture was centrifuged for 13 hours at 40,000 rpm in a Spinco Preparative Centrifuge (Model L), yielding a gel-like bottom portion which contained approximately half of the total protein while the remainder was in the fluid upper portion. The gel-like portion in the bottom third of the tube was removed and resuspended in saline. Three additional cycles of centrifugation and resus-

§ Independent experiments with the urinary Bence-Jones protein of this patient and human gamma globulin (Lederle), showed that under these conditions only the Bence-Jones protein precipitated, whereas the gamma globulin remained in solution.

‖ When the urinary Bence-Jones protein of this patient was allowed to interact with the rabbit anti-human gamma globulin, under identical conditions, only a trace of precipitate was formed.

TABLE I. Incorporation of C^{14} into Cell-free Non-dialyzable Part of Culture Fluid.

Sample	Counts per min for infinite thickness
Culture Fluid 72 hr	1990
" " 168 "	2120
Reservoir Medium	54.1

pension of the sedimenting material were carried out, with the time for centrifugation for the 3rd and 4th cycles reduced to periods of 8 and 7 hours respectively. The lower portion of the contents of the final tube was then removed for analysis.

The fluid upper portion obtained from the first centrifugation was diluted with 5 ml of saline and centrifuged for 12 hours at 40,000 rpm. The upper two-thirds was removed for analysis. Aliquots were taken from this sample, as well as from the preparation obtained after 4 cycles of high speed centrifugation, for protein determination and for measurement of radioactivity. To determine radioactivity, the proteins were precipitated with trichloroacetic acid at 100°C, washed 3 times with distilled water, resuspended in 0.02N NaOH, and counted.

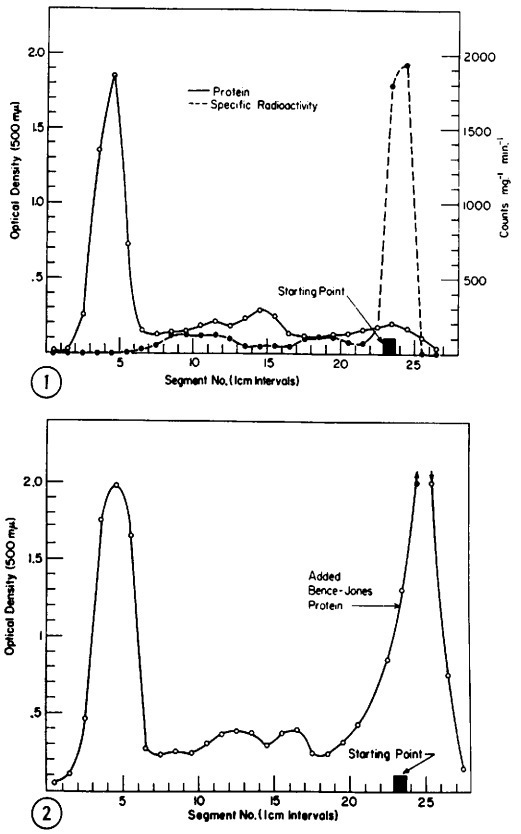

FIG. 1. Starch zone electrophoresis of dialyzed culture fluid and distribution of radioactivity.

FIG. 2. Starch zone electrophoresis of dialyzed nutrient medium containing added Bence-Jones protein.

The remainder of these 2 samples were dialyzed against Veronal buffer and centrifuged in the Spinco Analytical Ultracentrifuge (Model E); they showed the expected sedimentation characteristics of Bence-Jones protein and gamma globulins.

Results and discussion. In the 72-hour cell-free dialyzed culture fluid a significant amount of C^{14} radioactivity was found as compared to the control sample of the reservoir medium similarly dialyzed (Table I). That the 168-hour sample showed only an insignificant increase in radioactivity as compared to the 72-hour sample could be explained by the fact that incubation for periods longer than 72 hours caused disintegration of the cells, as shown by histological examination.

To identify the components which contained C^{14} radioactivity, the dialyzed material from the 72-hour culture fluid was subjected to fractionation by starch zone electrophoresis. Fig. 1 shows that essentially all the C^{14} radioactivity is present in segments 23-25 which correspond to the region in which the gamma globulins and the patient's Bence-Jones protein (Fig. 2) are found.

To determine whether the C^{14} radioactivity isolated from the starch zone segments 23-25 was part of the Bence-Jones protein, the gamma globulins or some other non-dialyzable substances, 3 independent fractionation procedures (using unlabeled Bence-Jones protein isolated from the patient's own urine and human gamma globulins as carriers) were employed: (1) precipitation of a protein fraction which coagulated at 58°C and redissolved at 100°C (2) specific antibody precipitation of human gamma globulins; and (3) differential centrifugation.

In the heat-precipitation method, the Bence-Jones protein could be precipitated at 58°C and thus completely removed from the other proteins. In Table II the radioactivity

TABLE II. Distribution of Radioactivity in Proteins Isolated from Starch Zone Segments 23-25.

Protein	Fractionated by heat precipitation	Fractionated by immunological reaction*	Fractionated by differential centrifugation
	% of total radioactivity		
Bence-Jones Protein	91.4	97.8	90.8
Gamma Globulins	8.6	1.3	9.2

* 0.9% of the total radioactivity was found in the supernatants.

of each fraction is shown as per cent of the sum of total C^{14} content. Over 90% of the C^{14} was found in the Bence-Jones protein fraction.

In the second fractionation procedure the gamma globulins were specifically precipitated with antibody to human gamma globulins. Comparison of the radioactivity in the two fractions obtained showed that very little activity was present in the gamma globulins, whereas the fraction, containing the Bence-Jones protein, accounted for practically all of the radioactivity.

In the third method, using differential centrifugation, advantage was taken of the known differences in molecular weight between gamma globulins and Bence-Jones protein. The radioactivity designated as gamma globulins in Table II corresponds to the fast sedimenting component and that designated as Bence-Jones protein to the slow sedimenting component. Again the major portion of the total radioactivity appeared in the Bence-Jones protein.

In drawing conclusions from this experiment, it should be noted that only a single case of multiple myeloma has been studied. The results show that most, if not all, of the incorporated C^{14} radioactivity corresponds to the fraction having the properties of a Bence-Jones protein and that probably the bone marrow constitutes a site capable of carrying out such an incorporation. The possibility that the incorporation of C^{14} radioactivity occurred in cells derived from peripheral blood, which was present in the aspirated bone marrow sample, cannot be excluded. Since in this particular case no blood samples were available for analysis, it was not possible to determine whether the patient was synthesizing an abnormal gamma globulin, and the significance of the negligible radioactivity in the gamma globulins cannot be evaluated.

Summary. It was shown that bone marrow cells from a myeloma patient were capable of incorporating *in vitro*, C^{14}-lysine into a substance having the characteristics of a Bence-Jones protein.

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