A CHARGE DIFFERENCE BETWEEN AN INTRACELLULAR AND SECRETED MOUSE MYELOMA GLOBULIN

G. W. Notani, A. J. Munro and P. M. Knopf
The Salk Institute for Biological Studies
P. O. Box 1809, San Diego, California 92112

Received October 3, 1966

A charge difference has been detected between the intracellular and secreted G myeloma globulin synthesized in vitro by murine plasmacytomas. The site of the charge difference has been located on the papain-produced Fc-fragment of the heavy chain*.

Materials and Methods

The plasma cell tumors MOPC21 and Adj-PC5, kindly given to us by Dr. M. Potter of N.I.H., were transplanted into groups of Balb/c mice. For the experiments reported here, transplantation generations 63 to 65 for MOPC21 and generations 91 to 93 for Adj-PC5 were used. The myeloma protein produced by MOPC21 is a γG_{2a} and by Adj-PC5 is a γG_{2b}. Sera from the tumor-bearing mice were pooled and the myeloma globulins were purified by precipitation with 17% (w/v) sodium sulfate at room temperature, followed by fractionation by DEAE-cellulose chromatography (Knopf et al., 1966). These purified proteins were used as carriers in the purification of radioactive leucine-labeled MOPC21 and Adj-PC5 myeloma globulins. The following procedures were adopted for the isolation of intracellular and extracellular myeloma proteins. Tumors were removed, cut into small pieces and the cells gently dispersed by the method of Jacob and Bhargava (1962) in fortified Eagle's medium (Vogt and Dulbecco, 1963) minus leucine. Cell viability was determined by the method of Rotman and Papermaster (1966); about 40% of the cells were found to be viable. The cells were washed twice in leucine-free Eagle's medium and then suspended at a concentration of 3×10^7 cells/ml in the same medium. One portion of the cell suspension was incubated at 37^o with H^3-L-leucine at 5μc/ml (5 mc/μmole, New England Nuclear) for 4-6 hours. Similarly, a second portion was incubated with C^{14}-L-leucine at 2 μc/ml (200 μc/μmole, Schwartz Bio Research Inc.)

*Terms recommended by the Committee on Nomenclature of Human Immunoglobulins, Bull. Wld. Hlth. Org. (1964). 30, 447.

Purification of soluble intracellular (internal) myeloma proteins. After incubation, the cells were centrifuged at 3000 x g for 5 minutes at 4°, resuspended in RSB medium (0.01 M Tris, pH 7.5, 0.01 M KCl, 0.0015 M $MgCl_2$) containing 1% sodium deoxycholate and the suspension homogenized for 5 minutes. Deoxyribonuclese (Worthington Biochemical Corporation) was added and the mixture centrifuged at 12,000 x g for 20 minutes at 4°. The supernatant was passed through Sephadex G-100 in PBS (0.02 M potassium phosphate buffer, pH 7.0, in 0.9% NaCl) to remove low molecular weight material. Carrier myeloma globulin was added and the solution was brought to 17% (w/v) sodium sulfate. After stirring for 1 hour at room temperatur the precipitates were centrifuged, washed once with 17% sodium sulfate and resuspended in PBS. Undissolved material was removed by centrifugation and the supernatant stored at -20°. Approximately 7% of the radioactivity in the Sephadex gel filtrate was recovered in this fraction.

Purification of extracellular (external) myeloma globulins. The incubated cell suspension was centrifuged at 3000 x g for 5 minutes at 4°. Carrier myeloma globulin was added to the supernatant, followed by sodium sulfate to a concentration of 17% (w/v). The precipitate was separated by centrifugation and dissolved in PBS. Approximately 60% of the trichloracetic acid precipitable activity present in the supernatant was recovered in the sodium sulfate precipitate.

The intracellular and extracellular myeloma globulins were combined at this stage for use in the subsequent experiments. In one experiment, C^{14}- labeled extracellular protein was mixed with H^3-labeled cells prior to extraction.

Chase experiment. Two 5 ml portions at 10^7 cells/ml were incubated, one with 5μc of C^{14}-leucine and the other with 12.5 μc of H^3-leucine. After 30 minutes incubation at 37°, the C^{14}-leucine labeled cells were centrifuged out and kept at 0°; to the H^3-leucine labeled cells was added a 1000-fold molar excess of unlabeled leucine and incubation continued for an additional two hours. Addition of unlabeled leucine completely quenched the uptake of H^3-leucine within 1 minute. The H^3-leucine labeled cells were centrifuged after the chase, combined with the C^{14}-leucine labeled cells, and the intracellular protein prepared as described above.

Separation of Heavy and Light Chains. Preparation of heavy and light chains was carried out by a modification of the procedure of Fleischman, Pain and Porter (1962). The combined mixture of purified internal and extracellular myeloma globulins of MOPC21 was reduced in 0.05 M dithioerythritol at pH 8.5 and alkylated with 0.15 M iodoacetamide. The mixture was loaded directly onto Sephadex G-100 equilibrated

with 1 M propionic acid and the separation of the globulin into heavy and light chains carried out at 4^0.

Papain digestion. Papain digestion of mixture of intracellular and extracellular myeloma globulins was carried out using a two-step procedure (Cebra et al., 1961) as modified by Knopf (details to be published). The protein was incubated with cysteine-free activated papain (weight ratio 700:1::γG:papain) for 5 minutes at 25^o, pH 8.0. The papain was inactivated with iodoacetamide (10^{-3}M) and removed by passage of the incubation mixture over Sephadex G-75. The intact myeloma globulin was then incubated for 90 minutes at 37^o with 0.2M mercaptoethanol and dialyzed against 0.01M potassium phosphate pH 8.0, 0.001 M mercaptoethanol. The fab-and Fc-fragments were fractionated on DEAE-cellulose using a gradient of phosphate buffer, pH 8.0, from 0.01 M to 0.3M.

Polyacrylamide gel electrophoresis. Protein mixtures were electrophoresed on 4.5 or 7.5% polyacrylamide gel in Tris TEMED buffer, pH 9.2. The 4.5% gels were prepared in buffer containing 7 M urea. Electrophoresis was carried out for 70-80 minutes at 5 ma/tube in Tris-glycine bridge buffer, pH 8.4. Fractions (1/32 ml) were collected by extruding the gel through a 1 ml syringe (Maizel, 1966), eluted in 1 ml 8 M urea and suspended in 15 ml Bray's solution containing Cab-O-Sil (4% by weight). This fraction size was experimentally found to be reproducible to within $\pm$ 10% and over 80% of the radioactivity was recovered from the gel. Radioactivity was measured in two energy channels; the H^3 and C^{14} were determined after correction for the overlap.

Results

Electrophoretic comparison between the intracellular and extracellular myeloma globulins of Adj-PC5 (Fig. 1a) and of MOPC21 (Fig. 1b) on polyacrylamide gel revealed differences in the relative electrophoretic mobilities, the secreted protein migrating faster towards the anode than the intracellular material. This electrophoretic difference was also found when the internal and secreted proteins of MOPC21 were isolated together (Fig. 1c). The contamination of myeloma globulins by other radioactive proteins was not significant as shown by the immunological precipitation data in Table I.

To determine the nature of this electrophoretic difference, the mobilities of the light and heavy chains from MOPC21 internal and external proteins were analyzed. The results show that the light chains are not distinguishable from each other (Fig. 2a);

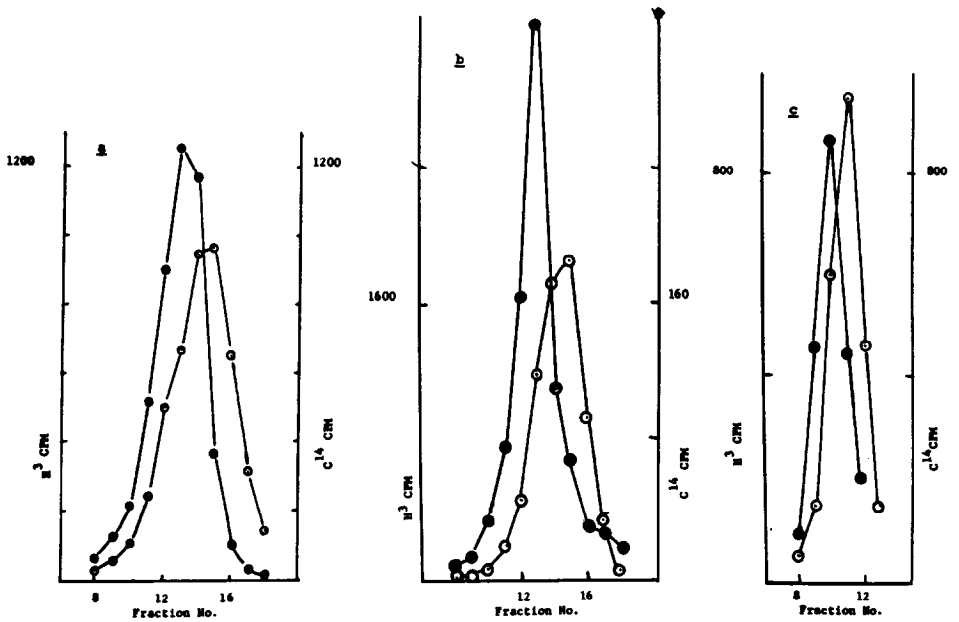

Fig. 1. Electrophoresis of myeloma globulin on 4.5% polyacrylamide.
(●-●) H^3-labeled intracellular γG
(0-0) C^{14}-labeled extracellular γG
(a) Adj-PC5; (b,c) MOPC21.

TABLE I

Precipitation of protein fractions with antiserum

	MOPC21		Adj-PC5			
	Internal γG (624 cpm)	Secreted γG (88 cpm)	Internal γG (1050 cpm)	Secreted γG (435 cpm)	Internal Fab (653 cpm)	Internal Fc (532 cpm
	cpm precipitated					
Anti-MOPC21 γG	573(93%)	79(90%)				
Anti-Adj-PC5 γG			1034(96%)	390(90%)		
Anti-Adj-Pc5 Fab					546(84%)	8(1.5%
Anti-Adj-PC5 Fc					10(1.5%)	512(96%)
Anti-ovalbumin			42(4%)			

Rabbit antisera against MOPC21 γG was absorbed with normal Balb/c serum. The anti-Adj-PC5 was prepared using a purified γG. The anti-Fc and Fab were prepared using Fc and Fab obtained by papain digestion method of Porter (1959). The anti-Fc was absorbed with Fab. Rabbit anti-ovalbumin was purchased from Hyland and used at equivalence with ovalbumin. All precipitations were carried out at equivalence using the appropriate carrier γG, by incubating 1 hour at 37° followed by 12 hours at 4°. Precipitates were washed once with saline and dissolved in 1 ml, 8M urea for counting.

the heavy chains, however, show an electrophoretic difference. The same result was found with Adj-PC5. Electrophoresis of a papain digest of the internal and secreted myeloma globulin mixture of Adj-PC5 (Fig. 2c) clearly shows the difference to be in the Fc-fragments. The Fab and Fc fragments were separated by DEAE-cellulose chromatography and each fraction electrophoresed on polyacrylamide. Again, the Fab fragments showed identical mobilities while the Fc-fragments were different. Identification of these fractions was established by specific antibody precipitations (Table I). The Fc-fragments of MOPC21 showed an electrophoretic difference and the Fab-fragments did not.

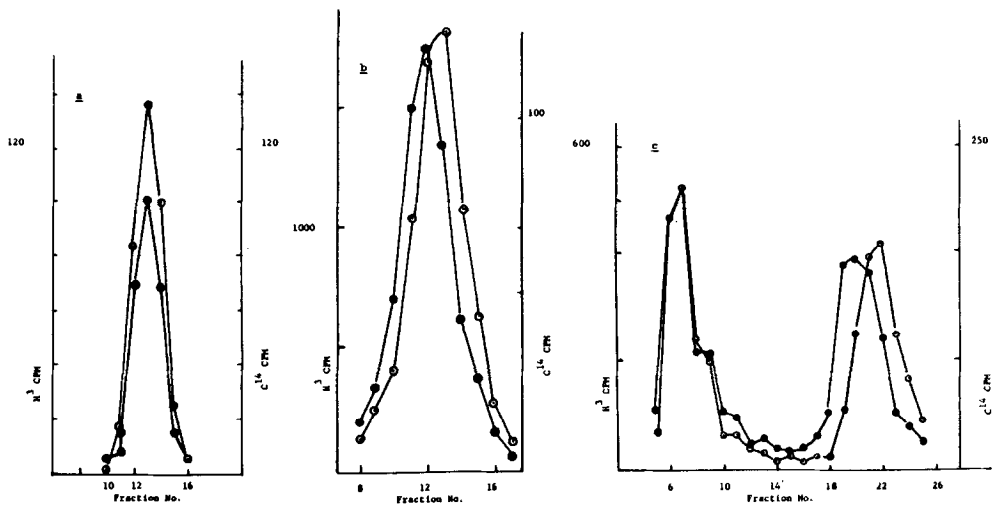

Fig. 2. Electrophoresis of Light and Heavy chains of MOPC21 γG on 4.5% polyacrylamide and Adj-PC5 γG papain digest on 7.5% polyacrylamide.
(●-●) H^3-labeled intracellular protein
(0-0) C^{14}-labeled extracellular protein
(a) MOPC21 light chains, (b) MOPC21 heavy chains, (c) Adj-PC5 γG papain digest.

The globulin isolated from cells labeled for 30 minutes with H^3-leucine followed by 120 minutes incubation with unlabeled leucine showed the same electrophoretic mobility as that from the cells labeled for 30 minutes with C^{14}-leucine (Fig. 3a). Hence the difference is conferred on the myeloma globulin either during or after its secretion from the cell. To distinguish between these two possibilities, purified intracellular Adj-PC5 myeloma globulin was incubated with Adj-PC5 cells in Eagle's medium for 4 hours at 37°. The incubated protein was combined separately with internal or external protein and electrophoresed on polyacrylamide. The results (Fig. 3. b,c) show that the soluble internal protein is not modified by incubation with the cells.

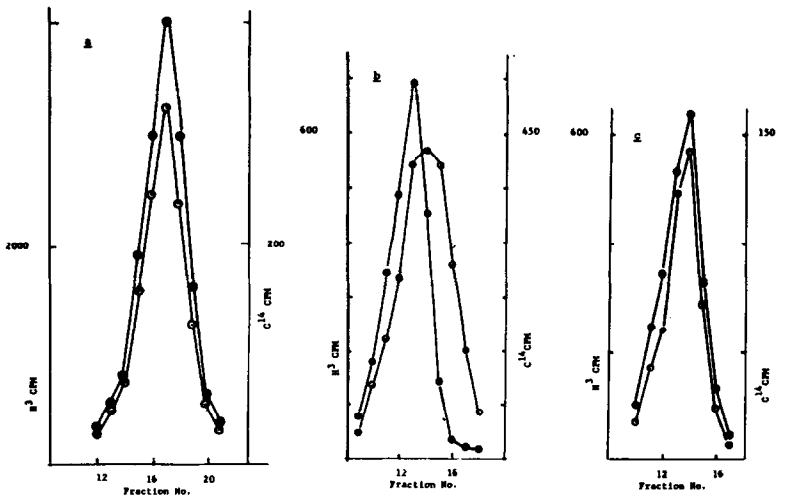

Fig. 3. Electrophoresis of myeloma globulin on 4.5% polyacrylamide.
(a). MOPC21 intracellular γG
(●-●) incubation for 30 minutes with C^{14}_3-leucine
(0-0) incubation for 30 minutes with H^3-leucine, followed by 120 minutes incubation with unlabeled leucine.
(b,c). Adj-PC5 γG
(●-●) H^3-labeled incubated intracellular γG
(0-0) C^{14}-labeled extracellular γG (3b)
(0-0) C^{14}-labeled intracellular γG (3c)

Discussion

The difference in electrophoretic mobility between intracellular and extracellular myeloma globulin has been located on the Fc-fragment. We attribute this difference in the mobility to a charge difference rather than to a change in the conformation of the protein because the heavy chain also exhibits the electrophoretic difference in 7 M urea. The result of the chase experiment tends to rule out the possibility of any slow process which converts the soluble internal myeloma globulin to the external form while it is still inside the cell. The mechanism by which charge(s) could be added or subtracted is not known, but the alteration would appear to be conferred upon the protein on its secretion from the cell.

The authors wish to thank Drs. Melvin Cohn and Edwin Lennox for their encouragement throughout these studies. This work was supported by National Institutes of Health Grants: AI05875 to Dr. Cohn, and AI-06544 to Dr. Lennox.

References

Bray, G. A., Anal. Biochem. (1960) 1, 279
Cebra, J. L., Givol, D., Silman, H. I. and Katchalski, E. (1961) J. Biol. Chem. 236,1720
Fleischman, J. B., Pain, R. H. and Porter, R. R. (1962) Arch. Biochem. Biophys. Supplement 1, 174
Knopf, P. M., Munro, A. J. and Lennox, E. (1966) Manuscript in preparation
Maizel, J. V. (1966) Sci. 151, 988
Porter, R. R. (1959) Biochem. J. 73, 119
Rotman, B. and Papermaster, B. W. (1966) Proc. Nat. Acad. Sci. 55, 134
Vogt, M. and Dulbecco, R. (1963) Proc. Nat. Acad. Sci. 49, 171