Carboxy-Terminal Amino Acids of γA and γM Heavy Chains

Abstract. *The carboxy-terminal amino acids of α- and μ-chains from human immunoglobulins and α-chains from mouse immunoglobulins have been determined by carboxypeptidase digestion and hydrazinolysis. The data suggest the following carboxy-terminal sequences: human μ: Ala-Gly-Thr-Cys-TyrCOOH; human α: Thr-Cys-TyrCOOH; murine α: (Ileu, Cys)-TyrCOOH.*

The presence of both γA and γM (immunoglobulins A and M) as polymeric proteins and their susceptibility to reductive cleavage to monomeric units indicates that, in both immunoglobulins, polymerization occurs through disulfide bond formation. Because of similarities observed in amino acid sequences of the Fc fragment portion of the heavy chain (*1*) and light chains, a common ancestry for both chains has recently been postulated (*2*). Since in the light chains both kappa and lambda have a cysteine residue at or penultimate to the carboxy-terminus which is involved in their disulfide linkage to heavy chains, the presence of such a residue at the carboxy-terminal end of α- and μ-chains was suspected, and experiments with carboxypeptidase digestion of a pathologic μ-chain indicated a sequence for the COOH-terminal dipeptide of Tyr-Cys or Cys-Tyr (*3*). We report experiments relating to the amino acid sequence in the COOH-terminal region of mouse and human α-chain and, for comparative purposes, experiments relating to human μ-chains.

Human γA and γM proteins were obtained from serums of patients with multiple myeloma and Waldenström's macroglobulinemia, respectively. Mouse γA was obtained from Balb/c mice with plasma cell tumors (*4*). Proteins were isolated by starch-block electrophoresis at *p*H 8.6 and purified by gel filtration through Sephadex G-200 in phosphate-buffered saline, *p*H 7.2. Mouse serums with high concentrations of both polymer and monomer, as judged by starch-gel electrophoresis, were selected. The isolated proteins were reduced with 0.2*M* 2-mercaptoethanol in 0.5*M* tris, *p*H 8.2, for 1 hour at room temperature and alkylated with a 10 percent excess of iodoacetamide in 0.55*M* tris for 1 hour at 4°C. Heavy and light chains of human γA and γM were separated by gel filtration on a G-100 Sephadex column equilibrated with 1*M* propionic acid (*5*). Attempts to separate heavy and light chains from reduced and alkylated mouse myeloma proteins by the same procedure were unsuccessful. However, this was accomplished by gel filtration through Sephadex G-200 equilibrated in 8*M* urea, 1*M* acetic acid, *p*H 3.6. The separated heavy chains were dialyzed against distilled water and, finally, against 0.1*M* ammonium bicarbonate. Carboxy-terminal amino acids of the isolated heavy chains were determined by digestion with carboxypeptidase A (diisopropyl fluorophosphate-treated from Worthington Biochemicals) and hydrazinolysis. The enzyme was diluted to a concentration of 2 mg/ml with 1.0*M* ammonium bicarbonate buffer, *p*H 8.2, added to 0.25 ml of a 1 percent solution of heavy chains, to make a molar ratio of enzyme to substrate of 1 : 50, and incubated at 25°C for different periods of time. Hydrazinolysis was done by adding 0.5 ml of anhydrous hydrazine to the previously dried proteins and incubating the samples at 100°C for 8 hours (*6*). Amino acids released were quantitatively determined with a Beckman model 120-C amino acid analyzer. Corrections for destruction of amino acids during hydrazinolysis were made from recovery of a mixture of amino acids after treatment with hydrazine.

Results of carboxypeptidase digests of a human μ-chain are shown in Table 1. Tyrosine and carboxyamidomethyl cysteine (CAMC) appeared in appreciable quantities after 10 minutes. In some other experiments at early time intervals with other μ-chains, tyrosine definitely appeared before significant amounts of other amino acids (Table 1, data in parentheses). The appearance of amino acids released after carboxypeptidase treatment suggests the sequence Ala-Gly-Thr-Cys-Tyr. The occurrence of tyrosine as the COOH-terminal residue was confirmed by hydrazinolysis. However, the relatively low final yield of tyrosine, compared to CAMC and threonine after 120 minutes of digestion, was disturbing. This was noticed in one of ten preparations of α-chains, as well, where there was no release of COOH-terminal tyrosine, even after prolonged periods of digestion. The explanation for this result is unknown; however, the finding of a low yield of tyrosine in some preparations may help to explain the discrepancy between the present results and those of Doolittle *et al.* (*3*), whose data suggested the sequence of Tyr-Cys, rather than Cys-Tyr.

Data on the results of carboxypeptidase digestion of the heavy chains of human and mouse γA proteins is shown in Tables 2 and 3. Tyrosine appeared first in both proteins, followed by CAMC and threonine in relatively low yields in the human, and by CAMC and isoleucine in the mouse chains. The data obtained with the mouse chains does not allow a clear positioning of the second and third residues from the end, but in the case of the human the data suggest the sequence of Thr-Cys-Tyr. The occurrence of COOH-terminal tyrosine in the mouse α-chain was confirmed by hydrazinolysis. A

Table 1. Carboxypeptidase digestion of human μ-chains. Figures in parentheses represent data obtained from a different μ-chain.

Digestion (min)	Products [mole amino acid/mole heavy chain (*8*)]				
	Tyr	CAMC	Thr	Gly	Ala
10	0.44	0.16	0.04	0	0
20	.42 (0.80)	.25 (0.04)	.12 (0.04)	Trace	0
60	.42	.45	.37	0.12	0.05
120	.52	.77	.91	.51	.49

Table 2. Carboxypeptidase digestion of α-chains from human immunoglobulins.

Digestion time (min)	Amino acid residue (mole per mole of heavy chain)		
	Tyr	CAMC	Thr
15	0.58	Trace	0
60	.58	Trace	0
120	.62	0.11	0
480	.58	.11	Trace
1260	.63	.16	0.16

Table 3. Carboxypeptidase digestion of α-chains from mouse immunoglobulins.

Digestion time (min)	Amino acid residue (mole per mole of heavy chain)		
	Tyr	CAMC	Ileu
20	0.75	0	0
60	.81	0	0
120	.89	0.28	0.20

Table 4. Carboxy-terminal sequence homologies among human immunoglobulin polypeptide chains.

Chain	Sequence
κ	Asn-Arg-Gly-Glu-CysCOOH
λ	Ala-Pro-Thr-Glu-Cys-SerCOOH
μ	Ala-Gly-Thr- -Cys-TyrCOOH
α	Thr- -Cys-TyrCOOH
γ	Leu-Ser-Leu-Ser-Pro-GlyCOOH

COOH-terminal tyrosine was observed in all three murine γA proteins studied, including one in which monomer and polymer forms were isolated and analyzed separately where both had a COOH-terminal tyrosine. Six of seven human α-chains had a COOH-terminal tyrosine. As mentioned above, one preparation completely lacked tyrosine, for unknown reasons, although it is possible that this represents a real heterogeneity within the γA class and is a manifestation of an α-chain subclass (*7*).

Table 4 summarizes the data and shows, for comparative purposes, the COOH-terminal sequence of γG heavy chains and κ- and λ-chains. The two most striking findings are (i) the very close relationship between the α- and μ-chains, compared with that of the γ-chain and (ii) when the COOH-terminal pentapeptide of the μ-chain is compared to κ- and λ-chains and a deletion is inserted for the sake of showing maximum homology, it is seen that the μ-chain pentapeptide more closely resembles that of the λ-chain than that of the κ-chain. These homologies are consistent with the hypothesis previously mentioned, that both heavy and light chains evolved from a common ancestral gene.

CARLOS A. ABEL
HOWARD M. GREY*
Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California

References and Notes

1. Fc fragment refers to the crystallizable fragment obtained after papain digestion of γ-globulin. All the nomenclature used is that which was recommended as a result of a conference on human immunoglobulins sponsored by the World Health Organization [*Bull. World Health Organ.* **30**, 447 (1964)].
2. R. L. Hill, R. Delaney, R. E. Fellows, H. E. Lebovitz, *Proc. Nat. Acad. Sci. U.S.* **56**, 1762 (1966); S. J. Singer and R. F. Doolittle, *Science* **153**, 13 (1966).
3. R. F. Doolittle, S. J. Singer, H. Metzger, *ibid.* **154**, 1561 (1966).
4. Supplied by Dr. M. Cohn, Salk Institute, La Jolla, California.
5. J. B. Fleischman, R. H. Pain, R. R. Porter, *Arch. Biochem. Biophys. Suppl.* **1**, 174 (1962).
6. E. M. Press, P. J. Piggot, R. R. Porter, *Biochem. J.* **99**, 356 (1966).
7. H. G. Kunkel and R. A. Prendergast, *Proc. Soc. Exp. Biol. Med.* **122**, 910 (1966); D. Feinstein and E. C. Franklin, *Nature* **212**, 1496 (1966); J. P. Vaerman and J. F. Heremans, *Science* **153**, 647 (1966).
8. M. E. Lamm and P. A. Small, Jr., *Biochemistry* **5**, 267 (1966).
9. J. J. Cebra and P. A. Small, Jr., *ibid.* **6**, 503 (1967).
10. We thank Dr. R. Doolittle for his helpful advice. This is publication No. 213 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California. Supported by USPHS grant AI-07007 and AEC contract AT(04-3)-410
* Established investigator of the American Heart Association.

21 March 1967; revised 18 April 1967

Leukemogenic Activity of Ether-Extracted Rauscher Leukemia Virus

Abstract. *After rapid multiple extractions of mouse plasma virus with ether, the aqueous solution contained viral nucleoids that were infectious when inoculated intracranially into newborn BALB/c mice. The infectivity associated with the ether extract was not neutralized by the specific antibody prepared against the whole virus. No intact virus has been seen in these preparations. Treatment with ether completely removed the virus envelope from the particle and produced an apparently homogeneous preparation of viral nucleoids. After the extractions with ether, leukemogenic activity was inactivated by exposure to ribonuclease. The leukemogenic activity of the many-passaged Rauscher virus that has been propagated in tissue culture and that has low infectivity was also retained, and, in two experiments in which material was inoculated intracranially into mice, this activity appeared to have been enhanced by multiple extractions with ether.*

This study was undertaken as an attempt to extract biologically active ribonucleic acid (RNA) from Rauscher leukemia virus. RNA was extracted by various modifications of the phenol procedure of Gierer and Schramm (*1*), as well as by the ether-treatment technique of Notkins (*2*). The RNA prepared by either hot or cold phenol techniques was noninfectious, since all newborn and weanling BALB/c mice inoculated (subcutaneously, intramuscularly, intravenously, intraperitoneally, and intracranially) with each such preparation failed to develop the Rauscher leukemia syndrome during observation periods ranging from 9 to 14 months. However, newborn BALB/c mice developed the typical disease after intracranial inoculation with the aqueous phase of ether-treated Rauscher virus (*3*). This report presents some of the biological properties, after treatment of intact virus with ether, of the "infectious nucleoids" of mouse plasma virus and of virus cultivated and apparently attenuated in vitro (*4*). Morphological features of the preparations are also discussed.

Plasma was collected from viremic BALB/c mice, and the virus was concentrated tenfold by differential centrifugation similar to the method described by Moloney (*5*). The virus pellet was resuspended in 0.01*M* phosphate-buffered saline (PBS) containing $10^{-4}M$ ethylenediaminetetraacetic acid (EDTA), at *p*H 7.2, and 2 percent fetal calf serum, and was stored at −120°F (−84°C).

The procedure for ether extraction, similar to that described by Notkins (*2*), was as follows. Equal amounts of concentrated plasma virus and freshly chilled ethyl ether were mixed for 2 minutes in a Vortex mixer. The phases were separated by centrifugation at 2000 rev/min for 5 minutes in a refrigerated centrifuge. The interphase layer, probably containing insoluble lipoproteins, was then removed and discarded. This procedure was repeated, usually five or six times, until the interphase between the aqueous and ether layers cleared. Fresh ether was added after each extraction. Residual ether was removed by bubbling nitrogen slowly through the chilled aqueous layer.

Approximately 80 percent of the original volume was recovered after multiple extractions with ether and ex-

Table 1. Bioassay of nontreated and ether-extracted Rauscher plasma virus after intracranial inoculation into newborn BALB/c mice, each mouse receiving 0.02 ml of inoculum. PBS, phosphate-buffered saline.

Inoculum (mouse plasma virus)	Palpation		Death		Average wt. of spleen (mg)
	P/T*	Mean time (days)	D/T*	Mean time (days)	
Intact virus + PBS (1:1)	10/10	28	3/10	34	1170
Intact virus + ribonuclease (1:1)†	10/10	28	2/10	37	965
Ether-extracted virus (undiluted)	15/18	33	4/18	29	1281
Ether-extracted virus + PBS (1:1)	8/14	43	4/14	42	530
Ether-extracted virus + ribonuclease (1:1)†	0/10		0/10		96

* Number with palpable spleens or number dead over total number inoculated. † Final concentration of ribonuclease was 100 μg/ml.