Acta Chemica Scandinavica B 34 (1980) 707–714

Purification of Two Ribonucleases from Macrophage Culture Medium and Studies on Their Effect on Granulation-Tissue Fibroblasts

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Two ribonuclease activities have been isolated from macrophage culture medium. SDS-electrophoresis gave a molecular weight of 26 000 for both RNAases.

The two RNAases differ only slightly in their enzymic properties. They are optimally active at neutral and slightly alkaline pH, and are not activated by monovalent or divalent cations or by spermine. Cu(II), Zn(II), Mn(II) and heparine inactivate them but they are not affected by the RNAase inhibitor from rat liver. They both degrade RNA endonucleolytically to mono- and oligonucleotides. They react to synthetic polynucleotides, especially poly(C), but do not degrade the synthetic double-stranded RNA poly(I)·poly(C), or double or single-stranded DNA.

RNAase 1 inhibits DNA synthesis and increases degradation of RNA in granulation-tissue fibroblasts but RNAase 2 at the same concentration does not have these effects.

It has been suggested that extracellular RNAases are endocytosed by intact animal cells and inhibit cell proliferation.[1-5] RNAases are involved in the regulation of the cellular RNA metabolism and changes in their activities have been reported during various conditions.[6-9] Macrophages have been observed to secrete lysosomal hydrolases[10] and also acid ribonuclease[11] to their culture medium in the presence of various agents. Also in the pulmonary tissue and in the air spaces macrophages normally lyse some of the protein[12] and especially collagen.[13] Through these secreted factors macrophages play an important role in inflammation and infection.[14,15]

There have been few studies on the molecular effects of these factors on cellular RNA metabolism. We have previously found that granulation-tissue RNA is rapidly degraded by the RNAases of macrophage culture medium.[16,17] This work deals with the isolation and characterization of this RNAase activity. A preliminary abstract has been published.[18]

EXPERIMENTAL

Materials. Calf thymus DNA (Type I), yeast RNA (Type II) and bovine serum albumin were obtained from Sigma Chem. Co., U.S.A. Before use the RNA was dialyzed for 24 h against 10 mM EDTA, against 0.15 M NaCl for another 24 h and finally for 24 h against water. Synthetic polynucleotides [poly(A), poly(C), poly(U), poly(G), poly(CG), poly(AC), poly(CU), poly(AU), poly(AGU) and poly(I)·poly(C); Cat. Nos. 108 626, 108 707, 108 928, 108 839, 108 723, 108 642, 108 731, 108 693, 108 685 and 108 898, respectively] were from Boehringer GmbH, Mannheim, West Germany. *p*-Nitrophenyl phosphate (disodium salt) was from Merck, Darmstadt, Germany, and bis-*p*-nitrophenyl phosphate from Sigma Chem. Co. U.S.A. Protamine sulfate was from Fluka AG and CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The protein standards for molecular weight determinations were from BDH Chemicals Ltd., Poole, England and the ribosomal 23S, 16S and 5S RNA of *E. coli* which were used as RNA standards from Miles Laboratories Ltd., Lausanne, Switzerland.

Culture of macrophages. Nonelicited macrophages were harvested from Wistar albino rats by washing the peritoneal cavity with 0.9% NaCl solution containing 10 IU/ml heparin. The cells were collected by centrifugation at 500 *g* for 10 min and washed twice with serum-free Dulbecco's modification of

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Eagle's Minimum Essential Medium, buffered to pH 7.4 with HEPES and $NaHCO_3$ containing 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate. The cells were then suspended into fresh serum-free medium and transferred to cell culture flasks (N 1475, Nunc, Denmark) (10^8 cells/174 cm^2 in 30 ml of medium) and allowed to adhere for 2 h. Nonadherent cells were then removed by decanting the medium and fresh serum-free medium was added. The cultures were incubated at 37 °C in an atmosphere of 95 % air and 5 % CO_2. The medium was collected after two days, filtered through Millipore filter (0.22 μ) and dialyzed against two changes of 0.05 M phosphate-buffer pH 6.2.

Culture of fibroblasts. Fibroblasts from two-week old experimental granulation tissue of rat were isolated by treating the sliced granulomas with collagenase, trypsin and hyaluronidase.[19] Cells were cultured in the growth medium described above containing 10 % foetal calf serum (FCS, Flow Laboratories). When confluent, the cells were detached with 0.25 % trypsin in Ca(II), Mg(II) free Hank's solution, and then subcultured at 1:4 dilution. Cells were labelled in microtest plate (Nunclon N-1482/1-67008) for 24 h with 7.4 kBq of (*methyl*-^{3}H) thymidine or (5-^{3}H) cytidine (TRK.300 and TRK.198 from the Radiochemical Centre, Amersham) at cell passages 4 – 8. In the pulse-chase experiment the radioactive medium was removed, new medium with 0.16 units of macrophage RNAase 1 was added and the incubation continued for the indicated times. After incubation the cells were detached with 0.25 % trypsin, collected onto glass fibre discs (GF/C) with an automatic cell harvester (made in our workshop) and the radioactivity in the cells was determined as described by Rönnemaa and Doherty.[20]

Assays of enzymic activities. Ribonuclease activity was determined in a reaction mixture which contained 50 μl 1 M Tris-HCl buffer (pH 7.8), 50 μl 0.02 % bovine serum albumin and 50 μl 0.2 % yeast RNA or 50 μg polyribonucleotides in a total volume of 300 μl. After incubation at 37 °C for 1 h in a shaking water bath the tubes were placed in an ice-water bath and an equal volume of 20 mM $La(NO_3)_3$ in 12 % $HClO_4$ was added after 1 min to stop the reaction. The tubes were centrifuged at 3000 *g* for 20 min at 4 °C and 500 μl of the supernatant was diluted with 500 μl of water. The increase in the absorbance of 260 nm was measured. One unit of enzyme produced 1 mmol of acid soluble nucleotides per hour under these conditions.

Deoxyribonuclease activity was assayed by measuring the acid soluble material liberated from DNA under the same conditions as the ribonuclease activity.

Phosphomonoesterase and phosphodiesterase activities were determined using *p*-nitrophenyl phosphate and bis-*p*-nitrophenyl phosphate as substrates.[21]

Protein. Protein was measured by the method of Lowry *et al.*[22] with bovine serum albumin as standard.

DEAE-cellulose chromatography of oligonucleotides. Column chromatography of the ribonuclease digest was performed on a DE52 column (Whatman) (2.2 × 20 cm) with a linear gradient of 0 – 0.3 M NaCl containing 7 M urea.[23]

Disc electrophoresis. Disc electrophoresis of the proteins was carried out in 5 % polyacrylamide gels with 4.06 % crosslinking[24] using the Molecular Weight Marker for SDS Polyacrylamide Gel Electrophoresis (M.W. range 14 300 – 71 500) as reference. Electrophoresis of RNA was carried out in 3 % acrylamide gels[25] with *E. coli* ribosomal and transfer RNAs as molecular weight markers.

Purification of RNAases. All of the following procedures were carried out at 0 – 5 °C.

Step 1. The dialyzed macrophage medium was applied to a column containing 2 g of CNBr-activated Sepharose 4B (1 × 5 cm) to which 10 mg of protamine had been bound covalently according to Pharmacia instructions. The column was washed with 30 ml of 0.05 M phosphate buffer (pH 6.2) and 5 ml fractions were collected. Those having RNAase activity were pooled (pool 2) and rechromatographed (step 2). The bound ribonuclease was eluted with 0.1 M acetate buffer (pH 4.0) containing 1 M NaCl. Fractions of 1 ml were now collected. Those having enzyme activity were pooled (pool 1), desalted by dialysis against 0.05 M phosphate buffer (pH 6.2) and re-chromatographed (step 2).

Step 2. The unbound fraction and the desalted bound fraction from step 1 were separately rechromatographed on a column of 10 mg poly(G) covalently bound to Sepharose 4B according to Pharmacia instructions. After addition of the enzyme solution, the column was washed with 30 ml of 0.05 M phosphate buffer (pH 6.2). Fractions of 5 ml were collected. The bound ribonuclease was eluted with 0.05 M phosphate buffer (pH 6.2) containing 1 M KCl. Fractions of 1 ml were collected. Those having the enzyme activity were combined and desalted by dialysis. The RNAase activities from the fractions bound and unbound to the protamine-Sepharose 4B column were designated RNAase 1 and RNAase 2, respectively.

RESULTS

Enzyme purification

Macrophage RNAases bind to DEAE-cellulose and can be eluted with 25 mM NaCl. They also bind to phosphocellulose from which they can be eluted with 300 mM NaCl. Ion-exchange chromatography on protamine-Sepharose 4B removed

Table 1. Purification of the ribonucleases from macrophage culture medium.

Fraction	Volume/ ml	Protein/ μg	Recovery of protein/ %	Activity/ units	Specific activity/ units μg^{-1}	Recovery of activity/ %	^{3}H-thymidine incorporation/ cpm/well[a]
Dialyzed macrophage medium	60	2880	100	43 632	15.2	100	1024(17)
Chromatography on protamine-Sepharose 4B							
Fraction (1) Bound	8.1	300	10	9 675	32.2	22	808(72)
Fraction (2) Unbound	61	2440	85	35 044	14.4	80	1304(117)
Chromatography on poly(G)-Sepharose 4B							
Fraction (1) Bound (ribonuclease 1)	6.5	58	2	9 534	164	22	680(16)
Unbound	15	255	9	–	–	–	1359(105)
Fraction (2) Bound (ribonuclease 2)	6.5	33	1	4 892	148	11	1347(53)
Unbound	68	2380	83	–	–	–	1330(112)

[a] The effect of a sample of 10 μl was tested on the ^{3}H-thymidine incorporation by granulation-tissue fibroblasts. Averages ±SEM ($n=3$) are presented. Control 1345 (78) cpm/well.

the biologically active fraction from the macrophage culture medium. This step also removed part of the ribonuclease activity from the medium and thus was used without any preceding purification steps.

The purification results are summarized in Table 1. Macrophages released 34 % of their proteins into the culture medium during two days. The RNAase activity which bound to protamine-Sepharose 4B was completely recovered on the following poly-(G)-Sepharose 4B chromatography step. It constituted 22 % of the original RNAase activity. Only 14 % of the RNAase activity which passed through protamine-Sepharose 4B was recovered after chromatography on poly(G)-Sepharose 4B. The main part of the activity (67 %) was lost during this step.

The absorption spectra of the RNAases are presented in Fig. 1 and show that a nucleic acid component is attached to the enzymes. However, no distinct molecular weight RNA species were seen in acrylamide gel electrophoresis.

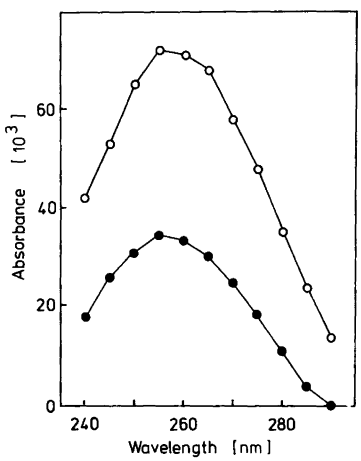

Fig. 1. Absorption spectra of macrophage RNAase 1 (○, 4 μg protein per 1 ml) and RNAase 2 (●, 2 μg protein per 1 ml) in 50 mM phosphate buffer (pH 6.2).

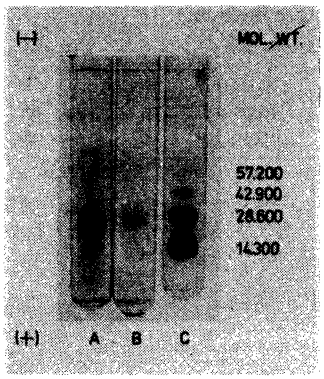

Fig. 2. Polyacrylamide gel electrophoresis of purified RNAases and molecular weight standards. A. 8 μg of RNAase 1; B. 4 μg of RNAase 2; C. molecular weight standard, 25 μl. Electrophoresis was carried out in 5% polyacrylamide gel in the presence of 0.1% SDS at pH 7.0 and the gels stained with Coomassie Blue.

Enzymic properies of the macrophage RNAases

Molecular weights of the ribonucleases. RNAase 1 and RNAase 2 migrate as single protein bands in polyacrylamide gels and the molecular weight is 26 000 for both (Fig. 2). Weak bands of 78 000, 104 000 and 130 000 molecular weights were sometimes observed, perhaps as traces of aggregates of 3, 4 and 5 enzyme molecules, and one band with 13 000 molecular weight, half of the main protein.

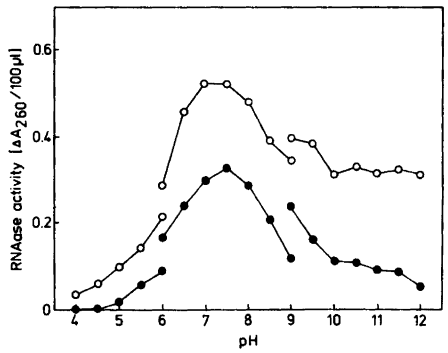

Fig. 3. Effect of pH on the activity of macrophage ribonucleases. For pH values ranging from 4.0–6.0 0.2 M succinate-borate buffer was used, for pH values ranging from 6.0–9.0 0.2 M phosphate-borate buffer and for pH values ranging from 9.0–12.0 0.2 M glycine–NaOH buffer was used. Reaction mixtures consisting of 50 μl buffer, 50 μl 0.02% bovine serum albumin, 50 μl 0.2% yeast RNA and 100 μl enzyme were incubated at 37 °C for 1 h. Acid soluble nucleotides were determined as described in Experimental. RNAase 1, 5 μg/ml (○), RNAase 2, 3 μg/ml (●).

Effect of pH on the activity of the ribonucleases. Both RNAases show maximal activity at neutral and slightly alkaline pH with an optimum at pH 7.5. There are no significant differences between the two RNAases (Fig. 3).

Effect of activators and inhibitors. The macrophage RNAases are not activated by NaCl, $MgCl_2$ or $CaCl_2$. High concentrations of these salts inhibit the activity of both RNAases (Table 2). The macrophage RNAases are sensitive to Zn(II), but not to Cu(II). They are not activated by spermine, which at higher concentrations is inhibitory. EDTA and *p*-chloromercuribenzoate have no effect on the activities.

Substrate specificity. The relative rates by which macrophage RNAases hydrolyze yeast RNA and polynucleotides are shown in Table 3. Macrophage RNAases degrade poly(C) to mono- and oligonucleotides with a K_m-value of 130 mg/l for both RNAases, but they do not degrade synthetic double-stranded RNA, poly(I)·poly(C). Neither do they show detectable activity on double and single stranded calf thymus DNA, *p*-nitrophenyl phosphate or bis-*p*-nitrophenyl phosphate.

Macrophage RNAases rapidly degrade the 28S and 18S rRNA from granulation tissue to fragments of 12S and 4S (Fig. 4). Both RNAases produce oligo-

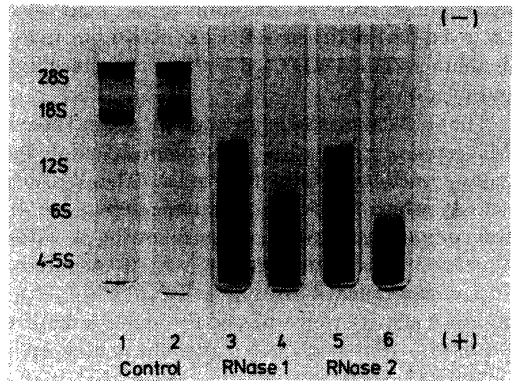

Fig. 4. Endoribonuclease action of purified RNAases on ribosomal RNA. 12 A_{260}-units of ribosomal RNA were incubated under standard assay conditions for 5 min (1,3,5) and for 60 min (2,4,6) without enzyme (1,2) with 4 units of RNAase 1 (3,4) or with 4 units of RNAase 2 (5,6).

Table 2. Effect of various inhibitors on the activity of macrophage ribonucleases. The reaction mixture for ribonuclease activity determination contained in a final volume of 300 μl: 50 μl 1 M Tris-HCl buffer (pH 7.8), 50 μl 0.02 % bovine serum albumin, 50 μl 0.2 % yeast RNA, 50 μl of the additions and 100 μl enzyme solution. After 1 h incubation at 37 °C the amount of acid soluble nucleotides was determined as described in the text.

Addition	Concentration/M	Ribonuclease activity/% of control activity	
		Ribonuclease 1	Ribonuclease 2
None	–	100	100
NaCl	1×10^{-2}	90	105
	1×10^{-1}	60	85
	5×10^{-1}	15	15
$MgCl_2$	1×10^{-3}	100	100
	1×10^{-2}	70	80
	5×10^{-2}	30	30
$CaCl_2$	1×10^{-3}	80	90
	1×10^{-2}	50	60
	5×10^{-2}	20	20
$CuCl_2$	1×10^{-4}	90	90
	1×10^{-3}	70	80
	1×10^{-2}	60	70
$MnCl_2$	1×10^{-4}	90	80
	1×10^{-3}	80	70
	1×10^{-2}	40	40
$ZnCl_2$	1×10^{-4}	80	70
	1×10^{-3}	30	40
	1×10^{-2}	20	20
Spermine	1×10^{-4}	70	80
	5×10^{-4}	60	70
	5×10^{-3}	30	30
Heparine	1×10^{-4}	50	70
	5×10^{-4}	40	40
	5×10^{-3}	30	30

and mononucleotides from yeast RNA, but the cleavage proceeds very slowly, as shown by DEAE-cellulose chromatography with 7 M urea (not shown).

Biological role of macrophage RNAases

Although both RNAases show similar enzymic properties they differ completely in their effects on cultured granulation-tissue fibroblasts. RNAase 2 had no effect on the thymidine incorporation into granulation-tissue fibroblasts, while RNAase 1 decreased the thymidine incorporation by 50 % (Table 1). During the purification of TNAases, only those fractions which contained RNAase 1 inhibited thymidine incorporation into granulation-tissue fibroblasts.

RNAase 1 had no effect on the degradation of DNA as measured by the release of labelled thymidine from cultured granulation-tissue fibroblasts, but it greatly accelerated the degradation of RNA as measured by the release of labelled cytidine from those cells (Fig. 5).

Table 3. Hydrolysis of synthetic polyribonucleotides by purified macrophage ribonucleases. Conditions for the determination of ribonuclease activity were as described in the legend for Table 2. The substrate was 50 μl 0.2 % yeast RNA in control samples (=100 %), and 50 μg synthetic polyribonucleotides in test samples.

Substrate	Ribonuclease activity/% of control activity	
	Ribonuclease 1	Ribonuclease 2
Yeast RNA	100	100
Poly(A)	30	20
Poly(C)	560	230
Poly(U)	40	10
Poly(G)	0	0
Poly(CG)	240	90
Poly(AC)	200	110
Poly(CU)	370	160
Poly(AU)	830	360
Poly(AGU)	190	100

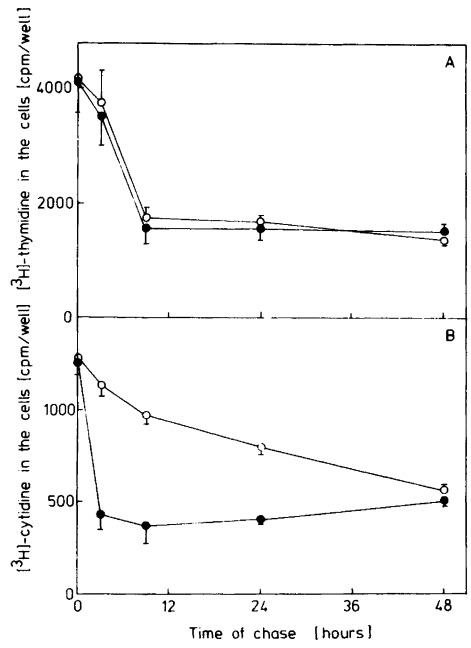

Fig. 5. The effect of RNAase 1 from the macrophage medium on the release of (A) (^{3}H) thymidine and (B) (^{3}H) cytidine from labelled granulation-tissue fibroblasts. The growing fibroblast cultures were labelled for 24 h with (^{3}H) thymidine, 7.4 kBq/well, and the confluent fibroblast cultures with (^{3}H) cytidine, 7.4 kBq/well, in medium containing 5% FCS. At the beginning of the chase, new medium without FCS and with or without RNAase 1 was added to the cells. The radioactivity in the cells was determined after various time periods as described in Experimental. No enzyme added (○), 250 ng of RNAase 1 (●). Averages ±SEM, $n=4$ are presented.

DISCUSSION

The behaviour of the macrophage RNAase during the purification. This work shows that macrophages in addition to their other functions[26] secrete RNAase activity into the culture medium. The amount of protein released from macrophages into the culture medium is in good agreement with our previous results.[27] On the basis of affinity the RNAase activity could be separated into two fractions, RNAase 1 and RNAase 2.

Ion-exchange chromatography with protamine-Sepharose 4B removed part of the ribonuclease activity from the medium. Because this fraction also contained the biological activity of the macrophage medium, this step was used without any preceding purification steps. RNAases have been purified by ion-exchange chromatography with *e.g.*, 5′(*p*-aminophenyl phosphoryl)-guanosine-2′(3′)-monophosphate,[28,29] concanavaline A,[30] ribonuclease inhibitor from rat liver[31] or poly(G)[32,33] bound to Sepharose 4B. The RNAase activity of the macrophage medium did attach to poly(G)-Sepharose 4B but not to the other ones mentioned above. The purified RNAases constituted 1 – 5 % of the total protein of the macrophage medium.

Properties of the purified RNAases. UV-absorption spectra of purified macrophage RNAases suggest that they contain a nucleic acid component, which is negatively charged and thus has affinity for positively charged molecules. This component may have a function in the RNAase enzyme activity. Other RNAases have also been reported to have an essential RNA component.[34–36]

The two RNAases differed only in their affinity properties and in their biological effects. Since Mg(II) and Ca(II) completely inhibited these enzymes and EDTA had no influence on their activity they cannot be phosphodiesterases.[37] In contrast to the observations that spermine increases the activity of poly(C) degrading enzymes,[38,39] spermine had only inhibitory effect on the macrophage RNAases.

The activity of the RNAases was measured as their ability to produce acid soluble nucleotides from yeast

RNA. However, a more likely role of macrophage RNAases is to degrade cellular RNA and by so doing to inhibit cellular protein and DNA synthesis. The RNAases rapidly degraded ribosomal 28S and 18S RNA to 12S and 4S fractions. Further degradation of these fragments to mono- and dinucleotides proceeded very slowly. The endonucleolytic degradation probably reflects the highly ordered secondary structure of rRNA with ribonuclease-sensitive pyrimidine rich regions at regular intervals.[40]

K_m values with yeast RNA or rRNA as substrates could not be determined, because cleavage of the long RNA-chains could not be determined quantitatively.

The enzymic properties of the macrophage RNAases resemble those of alkaline RNAase II from various tissues,[37] but macrophage RNAases preferentially hydrolysed poly(C) instead of poly(U). Alkaline RNAases from most tissues are associated with an endogenous inhibitor or they are sensitive to RNAase inhibitor from rat liver.[6,7,41,42] Rat liver RNAase inhibitor and also *p*-chloromercuribenzoate had no effect on the macrophage RNAases. McIntosh and Rabin[43] have also found an alkaline RNAase, which is not inhibited by the RNAase inhibitor.

The biological effect of the macrophage RNAase. Fetal calf serum, which is present in the fibroblast culture medium contains alkaline RNAase.[44] The serum RNAase activity in the fibroblast culture medium was found to be about tenfold higher than the macrophage RNAase activity. However, only the effect of the macrophage RNAase was observed on the RNA metabolism of the cultured fibroblasts. This may be due to the fact that different RNAases and their derivatives, especially dimerized ribonuclease A, are selectively taken into the cells.[1,2,4]

The purified enzyme had properties similar to the DNA synthesis inhibiting factor separated from macrophage culture medium by Toh.[45] Both are basic proteins which are separated into two fractions on the basis of charge, and have molecular weights of $(2-3) \times 10^4$. Macrophage RNAases may play an important role in the development of the granulation tissue during the early phase, at which stage there is a large amount of macrophages.[29,46,47] In the cultured fibroblasts, the effect of exogenous RNAases is primarily observed as increased RNA degradation and subsequently decreased DNA and protein synthesis.

Acknowledgements. I wish to thank Professor Eino Kulonen from the Department of Medical Chemistry, University of Turku, Turku, Finland, for useful discussions and help during the experiments and preparation of the manuscript. I am indebted to Miss Päivi Mäki for skillful technical assistance. A personal grant from the Emil Aaltonen Foundation and an institutional grant from the Medical Research Council of Finland are gratefully acknowledged.

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Received May 30, 1980.