High-Molecular-Weight Immunoreactive β-Endorphin in Extracts of Human Placenta Is a Fragment of Immunoglobulin G

Abstract. *A high-molecular-weight protein with β-endorphin- and adrenocorticotropin-immunoreactivities was isolated from extracts of human placenta after several purification steps, including immunoadsorption with a well-characterized antiserum raised to β-endorphin. This protein was identified as the heavy chain of the human immunoglobulin class IgG$_1$. These results have led to the recognition of homologies in the amino acid sequences of these physiologically unrelated molecules. They also suggest caution in accepting immunological competence as the sole criterion of the chemical identity of a ligand.*

The complete amino acid sequence of the precursor molecule (pre-pro- hormone) of the β-lipotropin (LPH), endorphins, adrenocorticotropin (ACTH), and melanotropins (MSH) has been reported by Nakanishi *et al.* (*1*), after cloning the isolated messenger RNA from bovine pituitaries. Prior to that time we had engaged in isolating the precursor molecule in amounts sufficient for amino acid sequencing, using extracts of human placenta. The presence of immunoreactive ACTH (*2*), α-MSH (*3*), and β-endorphin (*4*) had been reported in such extracts of human placenta. Moreover, we had access to unlimited amounts of side fractions from an industrial plant processing 9 to 12 metric tons of human placenta daily for the production of albumin and immunoglobulins of high purity (*5*).

Radioimmunoassays for β-endorphin (*6*) and ACTH (*7*) were performed exclusively on nondialyzable material, including heated controls for possible proteolytic activities. In the initial tissue extract (*5*), the amount of nondialyzable immunoreactive β-endorphin (ir-β-endorphin) accounted to 1.5 nmole per gram of wet tissue. As summarized in Table 1, 2450 kg of an industrial side fraction corresponding to 11,000 kg of tissue were processed, and 269 mg of a homogeneous protein were recovered as the only high-molecular-weight ir-β-endorphin in the starting material. Determination of molecular size by gel electrophoresis (*8*), amino acid analysis, and equilibrium sedimentation (*9*) were in agreement with a 48,500-dalton protein of about 420 amino acids containing 2.8 percent sugars. One-half of this material was hydrolyzed with trypsin and four tryptic peptides were purified. The tryptic digest had no opiate-like activity in the bioassay (guinea pig myenteric plexus-ileum) (*10*), as would have been expected

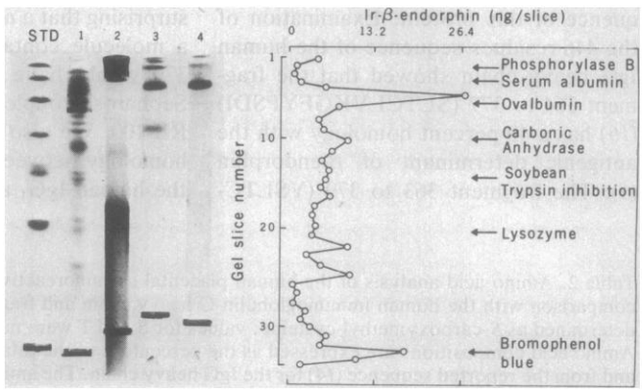

Fig. 1. Electrophoresis of placental fractions. Sodium dodecyl sulfate (SDS), urea, 7.5 percent polyacrylamide gels. Immunoreactivity of slices corresponding to gel 1. Gel 1 shows 50 μg of the material not retarded on DEAE-cellulose (step 4 in Table 1). Gel 2 shows 35 μg of the proteins bound to the immunoadsorbent and eluted with citrate buffer (step 5 in Table 1) under nonreducing conditions. Gel 3 shows the same as gel 2 after carboxymethylation. Gel 4 shows 20 μg of the final fraction (step 6 in Table 1). The samples were incubated as in (*8*) with 1 percent 2-mercaptoethanol, except for sample 2. The lower bars indicate migration of bromophenol blue. Three gels identical to gel 1 were cut into 2-mm-thick fragments; proteins were eluted by soaking the gels for 20 hours at 4°C in 1 ml of buffer C (*6*), used for radioimmunoassay, and assayed for ir-β-endorphin (*6*). Results are expressed in nanograms of β-endorphin per slice.

Table 1. Purification of the immunoreactive high-molecular-weight β-endorphin from human placenta. The hemoglobin residue (2450 kg, at present an industrial waste) from 24,000 liters of 25 percent alcohol supernatant (step 1), corresponding to 11,000 kg of placenta, was extracted with one volume of distilled water at *p*H 10.5 (NaOH). After removal of residual hemoglobin, the proteins (4850 g) containing immunoreactive β-endorphin (ir-β-endorphin) were recovered after precipitation with 3.1*M* ammonium sulfate by filtration through press filters (step 2). The extract was then fractionated with ammonium sulfate (step 3). The 250 g of proteins recovered after dialysis (cutoff, 8500 daltons) contained ir-β-endorphin eluting from Sephadex G-100 with K_{AV} : 0.045 in 1*N* acetic acid. The material from step 3 was processed in 125-g batches by ion-exchange chromatography. The bulk of the ir-β-endorphin was not retarded on the ion-exchanger at *p*H 7.75 (step 4). Electrophoresis (*8*) of this fraction on an SDS-urea-polyacrylamide gel is shown in Fig. 1, gel 1. Immunoadsorption (step 5) was completed in three separate runs, on a 300-ml column of the immunoadsorbent (100 ml of RB-100 antiserum coupled to Sepharose 6B) and the ligand was eluted with 0.05*M* sodium citrate, *p*H 2.8 (*20*). The total binding capacity of the column bed was calculated to be > 600 mg of β-endorphin. The immunoreactive β-endorphin proteins were dialyzed and lyophilized; after this treatment, the proteins were insoluble in aqueous buffers and did not migrate in nonreducing conditions on a 7.5 percent polyacrylamide gel (Fig. 1, gel 2). Reduction was followed by carboxymethylation (*9*) and resolved the aggregate in two main components (Fig. 1, gel 3). They were separated on Sephacryl S-200 superfine in a mixture of 5*M* urea and 0.5*M* ammonium bicarbonate, *p*H 7.0 (step 6). After gel electrophoresis, only the heaviest component was immunoreactive. The final fraction was homogeneous on gel electrophoresis (Fig. 1, gel 4) and equilibrium sedimentation in 6*M* guanidine. The radioimmunoassay for β-endorphin, which showed parallel curves of competition with ^{125}I-labeled human β-endorphin, was used to calculate recoveries and purification factors from steps 1 through 4. The amount of immunoreactive β-endorphin for step 6 was calculated on the basis of one determinant per 48,500-dalton molecule. Because the carboxymethylated protein could not be solubilized completely (see above), the radioimmunoassay on the final fraction is not quantitative.

Step	Fraction	Proteins (mg)	ir-β-Endorphin equivalents	
			β-Endorphin (μmole)	48,500-dalton protein (mg)
1	25 percent alcohol supernatant	360 × 10^6	22	1067*
2	Hemoglobin residue extract	4.85 × 10^6	2.36	114*
3	2.1 to 3*M* ammonium sulfate	250,000	2.46	119*
4	DEAE-cellulose eluate	20,000	1.09	52.8*
5	Immunoadsorption	1525		
6	Final	269		269†

*Theoretical weight based on immunoreactivity. †Actual weight of the final product.

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from a precursor of β-endorphin, since the fragment corresponding to β-LPH (fragment 61 to 69) should have been generated (*11*). The amino acid composition of the protein and those of the tryptic fragments, which are presented in Table 2, were then entered in a computer program (*12*) to search from homologies with the known polypeptide sequences compiled by Dayhoff *et al.* (*13*). The tryptic peptides from chorionic origin showed 80 to 100 percent homologies with several locations from the constant regions of the heavy chain of the human immunoglobin class IgG (*14*) as presented in Table 2. Furthermore, the sequence of peptide I was determined by mass spectrometry (*15*) as GPSVFPLAPSSK, which is identical to the fragment 122 to 133 for the heavy chain of the human IgG. The explanation for the purification of this aberrant protein in spite of the use of a well-characterized immune serum (*6*) in one of the most powerful and (supposedly) specific techniques, that is immunoadsorption, is probably found in the amino acid sequence of this protein: Examination of the 446 residues sequence of the human IgG heavy chain showed that the fragment 364 to 377 (SLTCLVKGFYPSDI) (*16*) has a 40 percent homology with the antigenic determinant of β-endorphin (*6*). The fragment 363 to 379 (VSLTCLVKGFYPSDIAV) was then synthesized by solid-phase techniques (*17*) and obtained in pure form. In standard radioimmunoassays, this human IgG fragment 363 to 379 showed an 0.18 percent displacement (on a molar basis) of the human β-endorphin tracer, with good parallelism (Fig. 2), when the antiserum RB-100 was used at a 1/35,000 dilution.

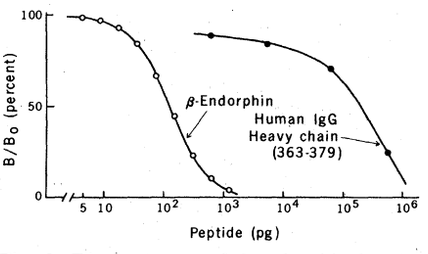

Fig. 2. Displacement of β-endorphin by the synthetic fragment 363-379 of human immunoglobulin G in the radioimmunoassay with the RB-100 immune serum.

Since in immunoadsorption the immune serum is used at a concentration up to 10^4 times greater than that used in the radioimmunoassay, it should not be surprising that a nonnegligible amount of a molecule containing fragment 363 to 379 would have been retained by the Sepharose-coupled immunoglobulins of RB-100. We also observed a 36 percent homology between the region 9 to 22 of the human IgG_1 and the sequence 11 to 24 of ACTH. Indeed, the final product of chorionic origin although barely soluble in the absence of concentrated urea or guanidine showed relative cross-reactivities of 0.039 and 0.0064 percent by weight (half maximal displacement), respectively, with the antiserum RB-100 (*6*) against β-endorphin and antiserum S_1B_2 (*7*) against ACTH. These ratios may reflect the difference in homologies (italicized residues) between fragments (363 to 379) and (9 to 22) of the immunoglobulin heavy chain, respectively V*S*L*T*C*L*V*K*G*F*YPSD*I*AV and AE*V*K*K*PGSS*V*K*V*SC, with the corresponding antigenic determinants of β-endorphin and ACTH (*6*, *7*), respectively K*S*Q*T*P*L*V*T*L*F*KNAI*I*KN and KP*V*G*K*KRRP*V*K*V*Y*P* for each of the antiserums (*18*).

These results raise (with some poignancy) the question of the ultimate specificity of analytical methods based solely or primarily on antigen-antibody combination in attempts at characterizing one ligand or another without chemical evidence of composition or primary structure. Just as statistical analyses do not affirm differences between populations but only affirm the absence of differences between samples of populations, methods based on immunoprecipitations, binding (as in immunocytochemistry or immunoadsorption), or

Table 2. Amino acid analysis of the human placental immunoreactive β-endorphin protein and four tryptic peptides derived from this protein; comparison with the human immunoglobulin G heavy chain and fragments. Amino acid analyses obtained after 20 hours hydrolysis (*9*). C was determined as *S*-carboxymethyl-cysteine; values for S and T were not corrected for destruction and W was determined spectrophotometrically. Amino acid compositions are expressed as the percentage of the total number of residues from the amino acid analysis for the placental protein and from the reported sequence (*14*) for the IgG heavy chain. The amino acid compositions of the tryptic peptides are expressed as the numbers of residues found per polypeptide chain, and the corresponding figures in parentheses are from the sequence of the IgG heavy chain (*14*). NH_2-terminal residues were obtained by the dansyl-Edman technique. One-letter symbols for amino acid residues are given in (*16*).

Amino acid	Amino acid (%)		Number of residues in							
	Placental protein	IgG γ-chain*	T1	IgG heavy (122 to 133)	T2	IgG heavy (307 to 317)	T3	IgG heavy (327 to 334)	T4	IgG heavy (361 to 370)
K	6.2	6.95	0.9	(1)	1.0	(1)	1.0	(1)	0.9	(1)
H	1.4	2.0			1.5	(1)			0.7	
R	2.9	2.46								
D + N	8.1	7.17			1.8	(2)			1.3	(1)
T	6.2	7.39			1.0	(1)			0.7	(1)
S	11.5	11.88	2.4	(3)					0.4	(1)
E + Q	10.1	9.64			1.0	(1)	1.0	(1)	0.9	(1)
P	10.1	8.29	2.8	(3)			2.3	(2)		
G	8.2	7.4	1.1	(1)	1.7	(1)			1.1	
A	4.3	4.93	1.0	(1)	0.6		1.7	(2)		
C	2.4	2.46							1.0	(1)
V	8.6	10.0	1.0	(1)	1.6	(1)			0.9	(1)
M	1.9	1.34								
I	1.9	2.24					0.9	(1)		
L	7.2	6.72	1.0	(1)	2.1	(2)	0.9	(1)	1.3	(2)
Y	3.8	4.0								
F	3.4	3.36	0.9	(1)						
W	1.3	1.56			1.4	(1)				
NH_2-terminal	(Z)	(Z)	G	(G)	T	(T)	A	(A)	N or T	(N)
			Numbers of residues							
	424†	(446)	12		12/13		8		10	

*From human serum EU. †Calculated on the basis of a 48,500-dalton molecule.

competition with a known ligand as in (radio)immunoassays will never allow affirmation of identity (of whatever is being investigated) with the known ligand or antigen involved. At best such methods can lead to affirmation of nonidentity, with the known ligand or antigen involved. At risk, they give a statistical security about certain relationship of whatever is being investigated with the spatial structure of the antigenic determinant of the antigen used in eliciting the immune globulins involved.

On a more constructive side our studies have led to the unexpected recognition of several homologies in the amino acid sequences of physiologically unrelated molecules such as a pituitary hormone and the constant chain of an immunoglobulin.

Note added in proof: After this manuscript was submitted for publication there appeared the report by Houck *et al.* (*19*) in which they conclude having obtained, in extracts of human placenta, a high-molecular-weight immunoreactive β-endorphin and two smaller molecules reactive in the radioimmunoassay and in a receptor-binding assay. The report by Houck *et al.* is a good example of the questions raised here. Other than the radioimmunoassay, there is no characterization of the high-molecular-weight material to justify its being referred to as the possible precursor of β-endorphin.

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A Nonadrenergic Vagal Inhibitory Pathway to Feline Airways

Abstract. *In cats anesthetized with chloralose-pentobarbital and artificially ventilated, electrical stimulation of the caudal end of the cut cervical vagus nerve has a biphasic effect on the bronchoconstriction induced by an intravenous infusion of serotonin. The response consists of a brief augmentation of bronchoconstriction followed by relatively prolonged bronchodilation. After muscarinic receptor blockade with atropine, vagal stimulation causes only bronchodilation. Vagally mediated bronchodilation is not affected by beta adrenergic blockade with propranolol, alpha adrenergic blockade with phenoxybenzamine, or adrenergic neuronal blockade with guanethidine, but is abolished by autonomic ganglionic blockade with hexamethonium. These findings support the conclusion that a nonadrenergic inhibitory nervous system is present in the pulmonary airways of the cat and that the system is supplied by preganglionic fibers in the cervical vagus nerves.*

Classical concepts of the nervous regulation of mammalian airway smooth muscle emphasize the importance of excitatory cholinergic nerves and inhibitory adrenergic nerves (*1*). However, the recent discovery of a third type of innervation in airway smooth muscle has brought about a revision of these traditional concepts. Several studies have demonstrated an inhibitory nervous system in the trachealis muscle of the guinea pig (*2–4*) and baboon (*5*) that is neither adrenergic nor cholinergic. An analogous system has been identified in isolated human airways from the trachea to the smallest bronchi (*6*). Because the transmitter has not been identified, the system is known as the nonadrenergic inhibitory system.

Although it has been postulated that nonadrenergic inhibitory fibers derive from the cranial parasympathetic outflow and travel to the lungs in the vagus nerves (*2*), direct evidence for such a pathway is lacking. We report here the results of a series of experiments in which we measured lung mechanics, electrically stimulated the cervical sympathetic and parasympathetic nerves under controlled physiological conditions, and administered selective pharmacologic blocking agents to demonstrate the presence of a nonadrenergic vagal inhibitory pathway to feline pulmonary airways in vivo.

We anesthetized 52 adult female cats intraperitoneally with α-chloralose (80 mg/kg) and sodium pentobarbital (5 mg/kg), cannulated the trachea, and applied positive-pressure ventilation (frequency, 30 breaths per minute; tidal volume, 13 cm^3/kg). Blood pressure was recorded from the femoral artery with a strain-gauge manometer (Statham P23 ID) and heart rate was monitored with a standard limb-lead electrocardiograph. To facilitate intravenous drug administration, we inserted polyethylene catheters into the right saphenous and brachial veins. All experiments were performed with the animals supine. Body temperature was maintained at 36° to 39°C by a heat-exchanging pad.

Airflow was determined by connecting

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