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DETECTION OF AN M_r 200,000 GLYCOPROTEIN IN THE CULTURE MEDIUM OF SKIN FIBROBLASTS FROM PATIENTS WITH HUNTINGTON DISEASE

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Huntington disease is a progressive neurological disorder with an autosomal dominant mode of inheritance. Using high resolution two-dimensional gel electrophoresis, we analyzed the intracellular and the released protein patterns of skin fibroblasts from HD patients and compared them to cells from apparently normal individuals matched for age and sex. No consistent differences were found in the pattern of total cellular proteins. In contrast, the culture medium from HD patients (12 of 19) contained an M_r 200,000 glycoprotein not found in twelve control cultures. The relation of this protein to the HD gene is unknown.

Huntington disease (HD) is a degenerative neurological disease characterized by movement disorder, personality changes and variable degrees of dementia (1). The onset of symptoms is usually between 30 and 50 years of age. The prevalence of recognized HD in the United States and Western Europe ranges from 4 to 7 per 100,000 population. The frequency of gene carriers is estimated to be approximately 1 in 10,000 (2-4). As an autosomal dominant trait, the offspring of an affected parent has a 50% chance of inheriting the disease. Since symptoms may not be recognized until the mid-thirties or later, individuals at risk usually have children without being aware of the risk to themselves or to their children.

The molecular defect in HD remains unknown. So far, there has been no reliable presymptomatic test to detect the carriers of the trait (5). Attempts

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have been made to search for protein markers for HD. The abnormal HD protein can arise either from the defective HD gene or it is caused by a secondary effect or it represents polymorphisms which are closely related with HD. Total intracellular protein from peripheral tissues, such as lymphocytes and skin fibroblast cultures have been examined by a number of investigators, yet no differences have been found so far (6-9). In this report we analyzed and compared the patterns of released proteins from normal and HD fibroblasts by two-dimensional gel electrophoresis. We found that HD fibroblasts released into the culture medium an extra protein of M_r 200,000. It is a glycoprotein with a pI around 6.8

MATERIALS AND METHODS

Cell cultures: Huntington disease fibroblasts and their age-matched controls were obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, New Jersey. HD cultures (GM 305, GM 1061, GM 1085, GM 1169, GM 1187, GM 2079, GM 2147, GM 2165, GM 2173, GM 3621); control cultures (GM 288, GM 322, GM 730, GM 2674, GM 2149, GM 2175) and four at risk individuals (GM 2151, GM 2171, GM 2177, GM 2183) were studied. Two HD lines (RL and VB) were provided by Dr. Andrew W. Zimmerman, University of Connecticut Health Center. In addition, six pairs of HD (4A, 5B, 6B, 7A, VDC, JD) and their controls (4B, 5A, 6A, 7B, FD, MD) were obtained from Dr. Ingeburg Goetz, City of Hope National Medical Center, Duarte, California. All cultures were studied between passages 4 and 14 (approximately 10 to 35 population doublings). Human foreskin fibroblast cultures derived from our laboratory at different passage levels were included as controls. Cells were grown in 10% fetal bovine serum (Gibco) in Dulbecco's Modified Eagle's Medium (DMEM) in 75 cm^2 flasks (Corning) passaged at split ratios of 1:3 and cultured at 37°C in 5% CO_2 in a humidified incubator. The cells were routinely checked for the presence of mycoplasma and were always negative.

Metabolic labeling: HD or control cell cultures at a density of approximately $1.2\text{-}2 \times 10^6$ cells/75 cm^2 flask were labeled with [^{35}S]-methionine (25 µCi/ml) (specific activity 1017 Ci/mmole, New England Nuclear in 10 ml of serum-free and methionine-free DMEM or with ^{14}C-total amino acids (5 µCi/ml, New England Nuclear) in serum-free DMEM for 7 h. At the end of the labeling period, the cells showed no morphological changes by phase contrast microscopy. The media were collected, centrifuged, dialyzed against 50 mM Tris-HCl (pH 7.2) with two changes and then lyophilized. After dialysis, the total media usually contained 6 to 8 $\times 10^5$ cpm. The cells were collected by scraping with a rubber policeman into ice-cold phosphate buffered-saline, centrifuged and stored as pellets at -20°C. Labeling of cells with [^{14}C]-glucosamine (1 µCi/ml, specific activity 355 mCi/mmol, New England Nuclear) was carried out in serum-free DMEM for 24 h. The cells and media were processed the same way as with [^{35}S]-methionine labeled samples. The radioactivity of the samples was measured by precipitating 10 µl aliquot with 10% ice-cold trichloroacetic acid (TCA) on Whatman 540 filter discs (2.4 cm). The discs were rinsed twice with 5% TCA and dried. Filters were dissolved in 0.5 ml of Protosol (New England Nuclear) and incubated at 55°C for 30 min and counted in scintillation fluid 949 (New England Nuclear) in a liquid scintillation counter.

Polyacrylamide gel electrophoresis: One-dimensional SDS gel electrophoresis was performed as described by Laemmli (10). The separating gel consisted of a 5-20% linear gradient and the stacking gel contained 3% acrylamide. The lyophilized media or frozen cell pellets were suspended in 250 µl of SDS gel sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.5% glycerol, 1.25% β-mercaptoethanol, 0.75% SDS) containing 8 M urea. The gel slab was electrophoresed at 50 mA constant current 1.5 h after the bromophenol blue dye front reached the bottom of the gel. The gel was fixed overnight, soaked in EN^{3}HANCE (New England Nuclear) for 4h and in water for 1h. The gels were dried on Biorad filter papers and exposed to Kodak X-Omat AR films for 7 to 30 days. The incorporation of radioactivity was measured by cutting the dried gels into 2 mm segments. Each segment was placed in a scintillation vial containing 0.5 ml of H_2O. Ten ml of liquid scintillation fluid 949 containing 3% Protosol was added to each vial. The vials were shaken at 37°C overnight and counted in a liquid scintillation counter.

Two-dimensional gel electrophoresis was carried out as described by O'Farrell (11). Thirty microliter aliquots of the samples (approximately 75,000 cpm corresponding to 1.2 ml of the original volume) were applied to the first dimension IEF gels (3 x 140 mm) containing 9.2 M urea, 2% nonidet P 40 (Particle Data Laboratories, Ltd), 1.6% Ampholine pH 5-7, 0.4% Ampholine pH 3-10 (LKB), 4% acrylamide. Colored pI markers (United States Biochemical Corp.) were electrophoresed in a separate tube to construct a pH standard curve. The first dimension gels were electrophoresed at 400 V for 16h and 800 V for 1h. The gels were equilibrated into SDS gel sample buffer for 1.5h with two buffer changes. The second dimension gel slab consisted of a 4% stacking gel and an 8% separating gel. The radiolabeled standard proteins (Biorad Laboratories) used in the second dimension were myosin, phosphorylase b, bovine serum albumin, ovalbumin, α-chymotrypsinogen and β-globulin (M_r 200,000, 92,500, 68,000, 43,000, 25,700, 18,400, respectively). The gels were prepared for fluorography as described before. The protein pattern of each sample was determined by at least two independent electrophoretic runs.

RESULTS

One-dimensional SDS gel electrophoresis was performed to analyze the [^{14}C]-amino acid labeled intracellular and released proteins from the skin fibroblasts of HD and control cultures. No obvious difference was found in the total intracellular proteins (results not shown). However, a high molecular weight protein M_r 200,000 was found in the medium of HD cultures but not in the control (Fig. 1). By measuring the radioactivity incorporated into this band, this M_r 200,000 protein was estimated to account for about 4% of the total protein in the medium. The [^{35}S]-methionine labeled HD cultures also showed this extra protein in the medium (results not shown).

In order to examine the possibility that the M_r 200,000 protein is related to cellular senescence, we analyzed the released protein pattern from human foreskin fibroblast cultures at different passage levels (passage 5, 15 and 45). We did not detect the presence of this M_r 200,000 protein in any of the culture medium (results not shown).

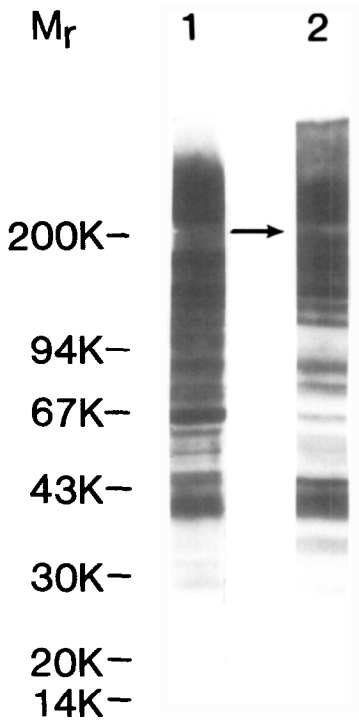

Fig 1. One-dimensional SDS polyacrylamide gel electrophoresis of proteins secreted by HD and control fibroblast cultures. Fibroblast cultures in 75 cm^2 flasks were labeled with [^{14}C]-total amino acids as described in Materials and Methods. The lyophilized medium was analyzed on a 5-20% gradient gel. Lane 1, GM 2149, a control culture; lane 2, GM 2147, an HD culture. The arrow indicates the M_r 200,000 protein.

We next analyzed the radiolabeled protein by two-dimensional gel electrophoresis. The pattern of [^{35}S]-methionine labeled whole cell lysate is shown in Fig. 2. Over three hundred spots could be resolved under our electrophoretic conditions. A number of quantitative differences between the HD and the control cultures were found, but these differences were not consistent from experiment to experiment.

Analysis of the released proteins by two-dimensional gel electrophoresis revealed approximately 60 spots. The M_r 200,000 protein was found in the medium of HD fibroblasts, GM 2147, GM 2079, GM 3621 (Fig. 3, panels A-C) but not in the control GM 2149 (Fig. 3, panel D). This protein appeared as a streak at pI 6.6-6.8. In addition, a number of other quantitative changes were observed. Cultures were also labeled with [^{14}C]-glucosamine and the released proteins were analyzed by two-dimensional gels. Approximately a dozen protein spots could be

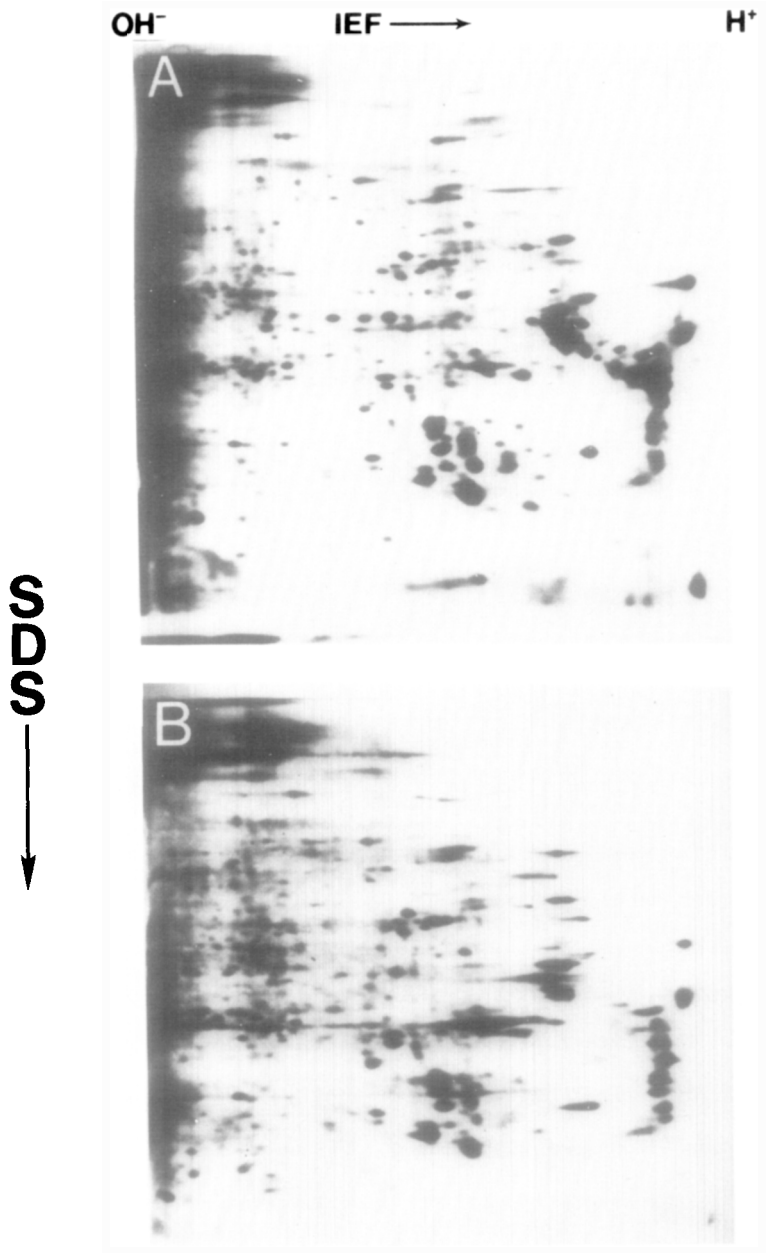

Fig. 2. Two-dimensional SDS polyacrylamide gel electrophoresis of total intracellular proteins of HD and control skin fibroblasts. Cultures of GM 1187 and GM 2175 were labeled with [^{35}S]-methionine in serum-free and methionine-free medium as described in Materials and Methods. The cell pellets were resuspended in Laemmli's gel sample buffer and two-dimensional gel electrophoresis was performed according to O'Farrell (11). Panel A, GM 1187, an HD culture; panel B, GM 2175, a control culture.

detected (Fig. 4). The M_r 200,000 HD protein was again found indicating that it was a glycoprotein (panel A).

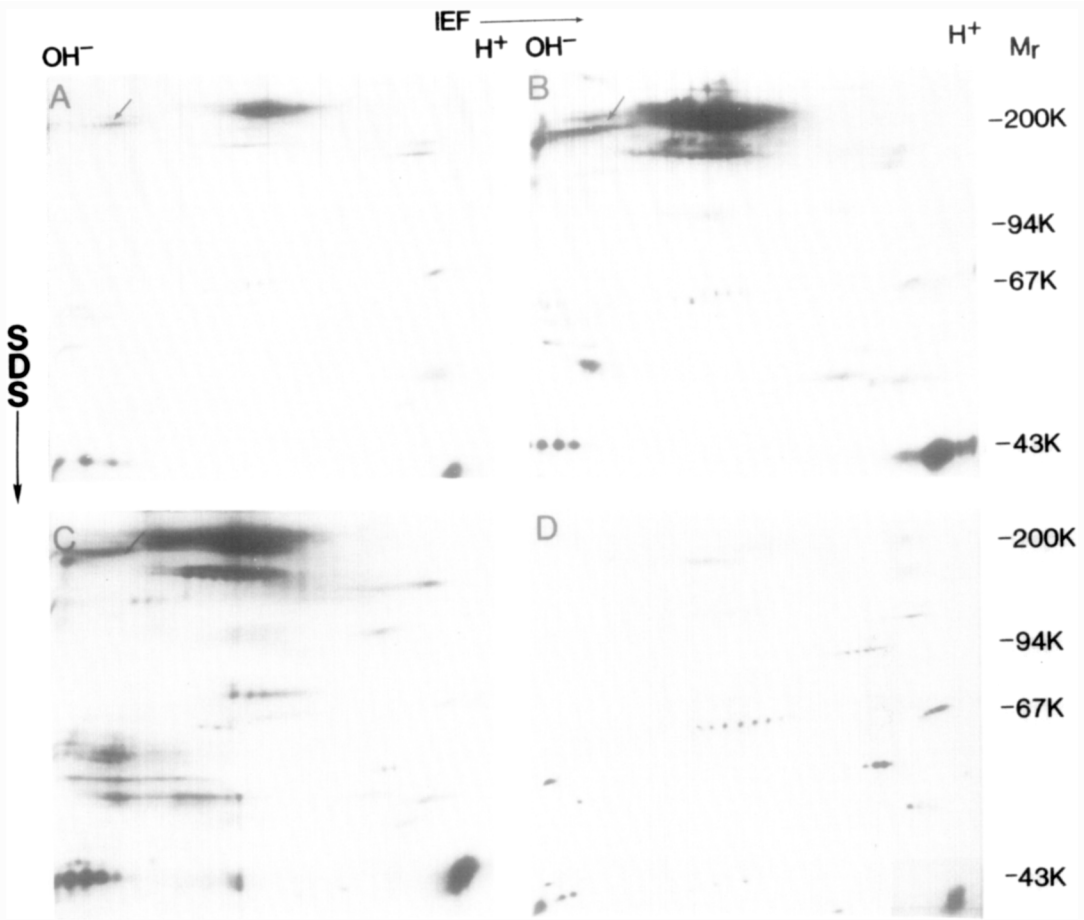

Fig. 3. Two-dimensional SDS polyacrylamide gel electrophoresis of released proteins from HD and control skin fibroblasts. Cells from three HD cultures GM 2147, GM 2079, GM 3621, and a control GM 2149 were labeled with [^{35}S]-methionine and the media were lyophilized and analyzed by two-dimensional gel electrophoresis. The M_r 200,000 glycoprotein is indicated by an arrow. Panel A, GM 2147; panel B, GM 2079; panel C, GM 3621; panel D, GM 2149.

Table 1 lists all of the skin fibroblast cultures that we have analyzed thus far. Twelve (4 males, 8 females) out of 19 HD cultures (10 males, 9 females) showed the M_r 200,000 glycoprotein. Quantitative differences were apparent among the HD strains. Among the three at-risk individuals studied, two clearly showed the extra band while the third showed a very faint band.

DISCUSSION

We chose to analyze the pattern of the proteins released into the culture medium by HD fibroblasts for several reasons. First, evidence has been given to

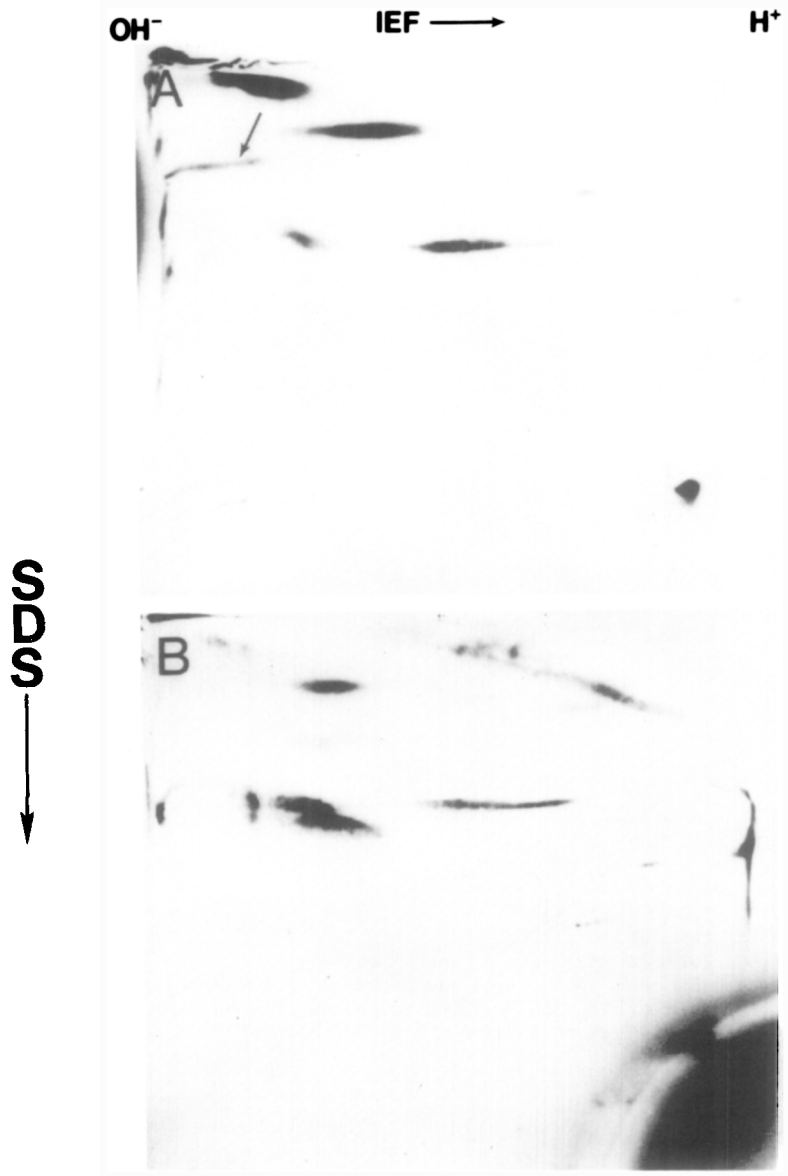

Fig. 4. Two-dimensional gel electrophoresis of proteins secreted by [^{14}C]-glucosamine labeled HD and control cultures. GM 2147 and GM 2149 cells were labeled with [^{14}C]-glucosamine and the lyophilized media were analyzed by two-dimensional gel electrophoresis. Panel A, GM 2147; panel B, GM 2149. Arrow indicates the M_r 200,000 glycoprotein.

suggest a membrane defect in HD fibroblasts (12). It seemed highly probable that a membrane defect could result in an alteration in the pattern of proteins released from fibroblasts. Second, secretory or released proteins are fewer in number, compared to intracellular proteins, and differences may be more readily

TABLE 1
ABUNDANCE OF THE M_r 200,000 PROTEIN IN HD, AT RISK AND CONTROL SKIN FIBROBLAST CULTURES

HD	Passage No.	Age	Sex	Abundance of M_r 200,000 protein
GM 2147	P14	55	M	+++
GM 2173	P8	52	F	++
GM 3621[1]	P13	29	F	+++
GM 1187	P10	43	M	-
GM 2079	P10	48	F	+++
GM 2165	P8	55	M	-
GM 1061	P6	51	M	-
GM 1169	P6	50	M	++
GM 1085	P7	44	M	-
GM 305	P13	56	F	+++
GM 2177[2]	P9	26	M	++
4A	P5	33	M	-
5B	P4	38	F	-
6B	P4	32	M	+++
7A	P9	51	F	+++
VDC	P6	61	F	++
JD[3]	P6	38	F	+++
RL	P7	45	M	-
VB	P9	69	F	+
At Risk				
GM 2151[4]	P10	28	F	+++
GM 2171[5]	P9	22	F	-
GM 2183[6]	P7	21	F	+++
Normal				
GM 2149[7]	P10	54	F	-
GM 2175[8]	P7	55	M	-
GM 322	P8	40	M	-
GM 288[9]	P13	64	M	-
GM 2674	P10	29	F	-
GM 730	P7	45	F	-
4B	P5	31	M	-
5A	P4	33	F	-
6A	P4	32	M	-
7B	P7	53	F	-
MD	P7	34	F	-
FD	P6	62	M	-

1 = daughter of GM 2165
2 = son of GM 2165
3 = daughter of VDC
4 = daughter of GM 2147, GM 2149
5 = daughter of GM 2173, GM 2175
6 = daughter of GM 2165
7 = wife of GM 2147
8 = husband of GM 2173
9 = husband of GM 305

detected. Among the proteins known to be released into the culture medium from human fibroblasts are fibronectin (13), collagen (14), collagenase (15), plasminogen activator (16), histocompatibility antigens (17), alpha-2 macroglobulin (18), a 100K glucose-regulated cell surface protein (19), a major fucosylated glycoprotein (20) and a 70K gelatin binding glycoprotein (21). None of these proteins corresponded in size to the M_r 200,000 glycoprotein found in the HD cell cultures.

The origin of the M_r 200,000 glycoprotein is unknown. Secretory or released proteins are known to undergo a number of post-translational modifications prior to their discharge from cells (for review, see 22). These modifications include limited proteolysis, glycosylation and disulfide bond formation (23-26). The M_r 200,000 glycoprotein may arise from (1) defective protein synthesis, (2) defective processing of a glycoprotein, (3) a mutation in the structural gene(s) for a glycosyltransferase or (4) defective degradation of a glycoprotein. Further chemical characterization of the glycoprotein is essential to identify the nature of the defect.

Of the nineteen HD cultures that we studied, twelve had the M_r 200,000 protein. One cell strain GM 2165, from an individual with HD, did not show the M_r 200,000 protein, while all three of his children, GM 2177 (HD), GM 3621 (HD) and GM 2183 (at-risk), had it. These findings suggest probable interfamilial and intrafamilial heterogeneity in the expression of the M_r 200,000 protein. In GM 2165 the protein may be present but in such a low level that it is beyond our current limit of detection. Alternatively, the protein may, in fact, be totally absent from the released protein of GM 2165. The latter possibility would indicate that the M_r 200,000 protein is not a direct product of the HD gene, but rather it represents a secondary or more distant effect.

We are now raising specific antibody against the M_r 200,000 protein in order to develop a sensitive immunological assay to detect the presence of this protein in a large number of HD and at-risk cell strains. The antibody could also be used for the isolation and characterization of the protein.

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REFERENCES

1. Huntington, G. (1872) Med. Surg. Reptr. 26, 317-321.
2. Myrianthopoulos, N.C. (1966) J. Med. Genet. 3, 298-314.
3. Myrianthopoulos, N.C. (1973) Adv. Neurol. 1, 149-159.
4. Shoulson, I., and Chase, T.N. (1975) Ann. Rev. Med. 26, 419-426.
5. Klawans, H.L., Goetz, C.G., and Perlik, S. (1980) Annal. Neurol. 8, 343-347.
6. Comings, D.E. (1979) Adv. Neurol. 23, 334-349.
7. Brown, W.T., Ambruster, J., and Darlington, G.J. (1979) Adv. Neurol. 23, 361-370.
8. Rosenberg, R.N., Ivy, N., Kirkpatrick, J., Bay, C., Nyhan, W.L., and Baskin, F. (1981) Neurol. 31, 1003-1014.
9. Goldman, D., Merril, C.R., Polinsky, R.J., and Ebert, M.H. (1982) Clin. Chem. 28, 1021-1025.
10. Laemmli, U.K. (1970) Nature 227, 680-685.
11. O'Farrell, P.H. (1975) J. Biol. Chem. 259, 4007-4021.
12. Tourian, A., and Hung, W.-Y. (1977) Biochem. Biophys. Res. Commun. 78, 1296-1303.
13. Hynes, R.O., Destree, A., Mautner, V.M., and Ali, I.U. (1977) J. Supramol. Struct. 7, 397-408.
14. Peterkovsky, B. (1972) Arch. Biochem. Biophys. 152, 318-328.
15. Stricklin, G.P., Bauer, E.A., Jeffrey, J.J., and Eisen, A.Z. (1977) Biochem. 16, 1607-1615.
16. Christman, J.K., Silverstein, S.C., and Acs, G. (1975) Plasminogen activator. In: Proteinases in Mammalian Cells and Tissues. (A.J. Barrett, ed.) pp 91-149, Elsevier North-Holland, Amsterdam.
17. Kapeller, M., Gal-Oz, R., Grover, N.B., and Dolijanski, F. (1973) Exp. Cell Res. 79, 152-158.
18. Mosher, D.F., and Wing, D.A. (1976) J. Exp. Med. 143, 462-467.
19. McCormick, P.J., Keys, B.J., Pucci, C., and Millis, A.J.T. (1979) Cell 18, 173-182.
20. Sear, C.H.J., Grant, M.E., and Jackson, D.S. (1977) Biochem. J. 168, 91-103.
21. Vartio, T., and Vaheri, A. (1981) J. Biol. Chem. 256, 13085-13090.
22. Redman, C.M., and Banerjee, D. (1980) Protein secretion and transport. In: Cell Biology: A Comprehensive Treatise, Vol. 4, (D.M. Prescott, L. Goldstein, eds.) pp 443-486, Academic Press.
23. Davidson, J.M., McEneany, L.S.G., and Bornstein, P. (1975) Biochem. 14, 5188-5194.
24. Schachter, H. (1974) Biochem. Soc. Symp. 40, 57-71.
25. Leblond, C.P., and Bennett, G. (1977) Role of the Golgi apparatus in terminal glycosylation. In: "International Cell Biology" (B.R. Brinkley and K.R. Porter, eds.) pp 326-336, Rockefeller Univ. Press, New York.
26. Anfinsen, C.B. (1973) Science 18, 223-230.