Differences in Proteins Synthesized by Fibroblasts from Normal Individuals and Patients with Complete Testicular Feminization

GAIL P. RISBRIDGER, B. A. K. KHALID, G. L. WARNE, and J. W. FUNDER,
Endocrine Clinic, Royal Children's Hospital, Parkville 3052 and Medical Research Centre, Prince Henry's Hospital, Melbourne 3004, Australia

ABSTRACT Patterns of protein synthesis by genital skin fibroblasts from three unrelated normal individuals and three unrelated patients with complete testicular feminization were compared by two-dimensional gel electrophoresis. Cell lines were maintained in monolayer culture and pulse labeled with [^{35}S]methionine. Cells were lysed in 9 M urea, and aliquots of 20 μl subjected to isoelectric focussing and polyacrylamide gel electrophoresis followed by autoradiography. Gels of control fibroblasts showed two proteins (mol wt $\sim$ 45,000, $\sim$85,000; $pK_i \sim 5.0$) markedly more prominent than on gels from affected fibroblasts. This pattern was unaltered by prior exposure to dihydrotestosterone, suggesting differences in constitutive proteins of the fibroblast cells. Parallel studies demonstrated a marked reduction in the ability of fibroblasts from patients with complete testicular feminization to bind androgens in vitro compared with those of normal individuals. The relationship between these proteins, androgen receptors, and androgen insensitivity requires further investigation.

INTRODUCTION

The syndrome of complete testicular feminization is characterized by a 46XY karyotype, bilateral inguinal, or abdominal testes, female-appearing external genitalia at birth, a blind vagina, and no Mullerian derivatives (1, 2). This androgen-insensitive form of male pseudohermaphroditism has subsequently been shown to be due to an abnormality in androgen receptor activity (3). Two distinct forms of such patients have been described by Amrhein et al. (4) on the basis of the binding of dihydrotestosterone (DHT)[1] to cultured fibroblasts from these patients: one type of patient has little or no cytosol binding activity while the other apparently has normal cytosol and nuclear binding.

In this study we have compared patterns of protein synthesis by genital skin fibroblasts from normal patients and those with testicular feminization. The newly synthesized proteins were resolved by isoelectric focussing and sodium dodecyl sulfate (SDS) electrophoresis (two-dimensional gel electrophoresis), a technique that permits the detection of structural rather than functional alterations in protein synthesis (5).

The aim of the study was: (*a*) to determine the degree of androgen insensitivity by comparing levels of methytrienolone (R1881) binding in genital skin fibroblasts from controls and affected patients; (*b*) to compare patterns of protein synthesis in fibroblasts from patients with testicular feminization and normal patients by two-dimensional gel electrophoresis; (*c*) to study the effect of exposure of DHT on the patterns of protein synthesis by both types of fibroblasts.

METHODS

Materials. (17α-methyl-^{3}H) methyltrienolone (17β-hydroxy-17α methylestra-4,9,11-trien-3-one, [^{3}H]R1881) 87 Ci/mmol and methyltrienolone (R1881) were obtained from New England Nuclear (Boston, Mass.). [^{35}S]methionine, >600 Ci/mmol, was obtained from Amersham, Corp., Amersham, England. The materials used for cell culture were those described by Choo et al. (6), and those for the two-dimensional gel electrophoresis as described by O'Farrell (5).

Cell culture. Fibroblast strains in these studies were established from explants of genital skin obtained from unrelated normal individuals (aged 0.5–7 yr) and three unrelated patients with complete testicular feminization (aged 2, 5, and 13 yr). Procedures for the maintenance and storage

Dr. Khalid is an Ansell (Australia) Research Scholar.

Received for publication 26 May 1981 and in revised form 17 August 1981.

[1] *Abbreviations used in this paper*: CSS, charcoal-stripped fetal calf serum; DHT, dihydrotestosterone; DMEM, Dulbecco's modified Eagles' medium; FCS, fetal calf serum.

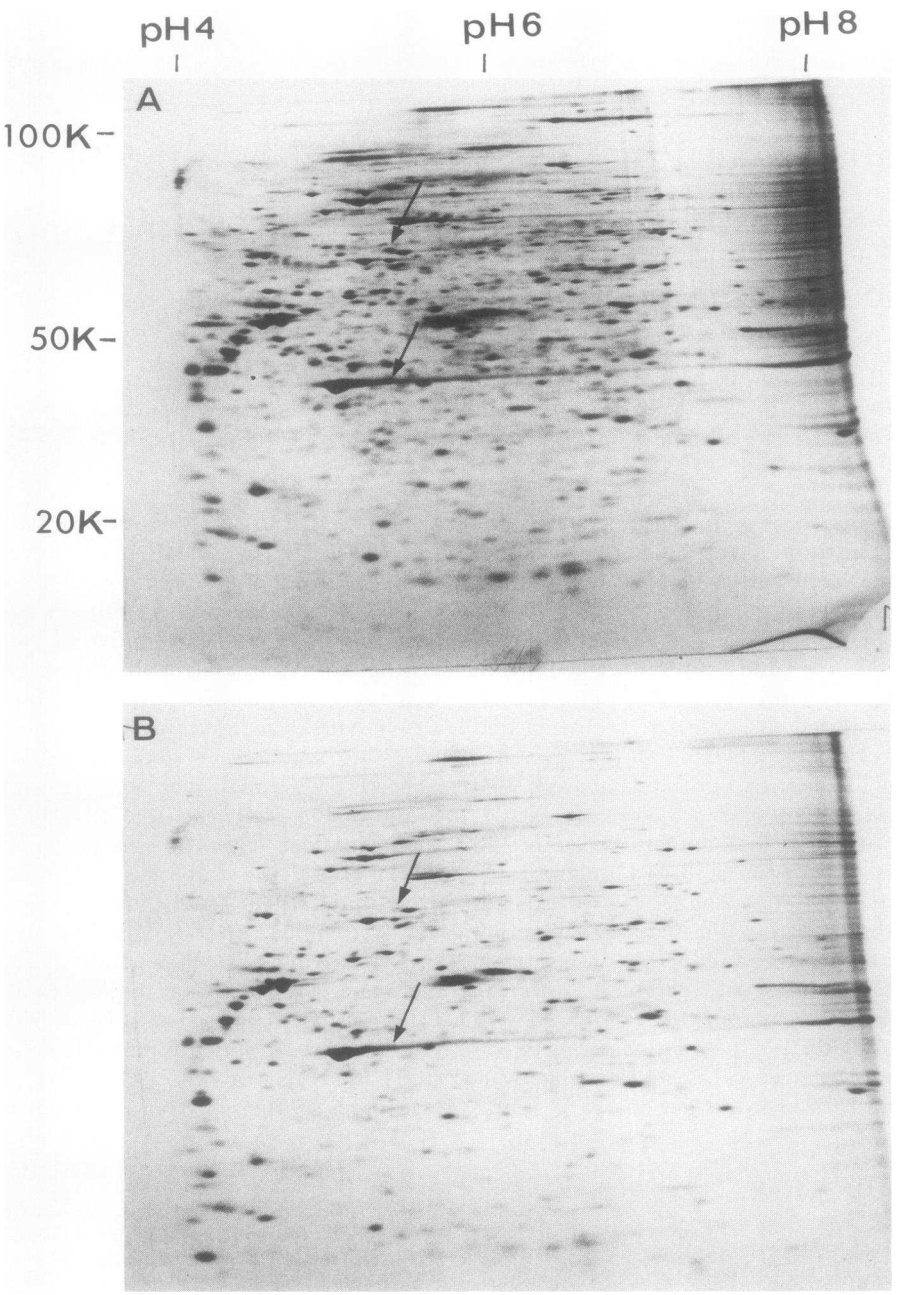

FIGURE 1 Pattern of proteins synthesized in serum-free media by genital skin fibroblasts from a normal individual, NC13 (Fig. 1A), and a patient with testicular feminization, Te4 (Fig. 1B). The pH gradient of the isoelectric focussing gel, and the position of the molecular weight markers are indicated in Fig. 1A. Arrows indicate the positions of two proteins more prominent in control fibroblasts than in patients' fibroblasts.

of these strains were as described by Griffin et al. (7). All fibroblasts were utilized between the 10th and 20th transfer.

DHT binding studies. To grow fibroblasts for the binding assays, cells from stock flasks were dissociated in 0.02% versene and 0.025% trypsin at 40°C, and transferred to roller bottles (285-mm Diam, Bellco Glass, Inc., Vineland, N. J.) in 50 ml Eagles' modified basal medium supplemented with 1 M Hepes buffer (2% vol/vol), polymyxin B sulphate (20 IU/ml), neomycin sulphate (10 IU/ml), and fetal calf serum (FCS) (10%), (FbGM + 10% FCS). The medium was changed as necessary, as the fibroblasts grew to confluency. 48 h before the experiment, the medium was replaced with that

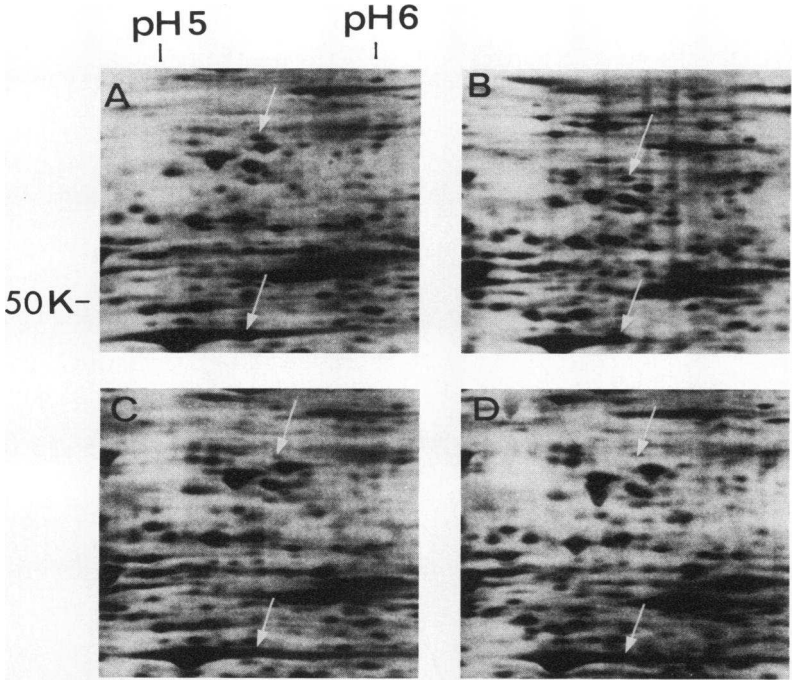

FIGURE 2 Pattern of proteins synthesized in serum-free media by fibroblasts from two normal (NC12 and NC14, Fig. 2A, C) and two testicular feminized individuals (Tela and Te3a, Fig. 2B, D). The samples NC12 and Tela were pulse labeled, and the newly synthesized proteins electrophoresed simultaneously. Similarly, but on a separate occasion, the cellular proteins from NC14 and Te3a were labeled and electrophoresed at the same time.

containing charcoal-stripped fetal calf serum (CSS) (FbGM + 10% CSS) to allow detection of the maximum binding capacity in the absence of endogenous steroids. The cells were then harvested from the roller bottles, and the DHT binding study performed on cell suspensions.

The cells were harvested by rinsing the cell surface with a Ca++-, Mg++-free phosphate-buffered isotonic saline solution at pH 7.4, and detaching the cells from the surface using a mixture of 0.02% versene and 0.025% trypsin in phosphate-buffered saline. Harvested cells were immediately placed in ice-cold buffer (0.02 M Tris-HCl, 0.32 M sucrose, 1 mM $MgSO_4$, pH 7.4 containing 1 mg/ml of human gamma globulin). The samples were centrifuged at 1,000 rpm for 10 min, the cells washed in 10 ml buffer (0.02 M Tris-HCl, 0.32 M sucrose, 1 mM $MgSO_4$, pH 7.4), and a cell count performed on an aliquot of this suspension. The cells were then resuspended to a concentration of 2×10^6 cells/ml in Dulbecco's modified Eagles' medium (DMEM). Triplicate aliquots of the cell suspension (0.5 ml) were incubated with 0.5 ml [^{3}H]R1881 (5 nM) for 60 min at 37°C in a 5% CO_2 incubator. Nonspecific binding was determined by the addition of a 100-fold excess of R1881 to a parallel set of tubes. The samples were then centrifuged at 1,500 *g* for 7 min at 4°C. After the final centrifugation the cells were resuspended in 1 ml DMEM and the [^{3}H]R1881 bound in a 0.9-ml aliquot determined in a Packard β counter (Packard Instrument Co., Inc., Downers Grove, Ill., model: B2450). The results were expressed as femtomoles of R1881 bound per million cells.

Pulse-labeling cellular proteins. For pulse-labeling studies, cells from stock flasks were dissociated with 0.2% versene and 0.025% trypsin at 40°C, and seeded ($0.5–1 \times 10^6$ cells) into wells of Linbro plates (60 mm Diam, Linbro Chemical Company, Hamden, Conn.) containing 10 ml FbGM + 10% FCS, and left for 1–2 d until confluent, as assessed by characteristic cell whorls. The medium was then replaced with FbGM × 10% CSS or FbGM + 10% CSS containing 50 nM DHT.

48 h later, the monolayer cultures were washed twice with DMEM with methionine. An aliquot of 2 ml of medium [DMEM without methionine, but supplemented with CSS (10%), 200 nM glutamine (1%), 1 M Hepes buffer (2%), 5.6% bicarbonate (2%)] was added to each well in the absence or presence of 50 nM DHT, together with 100 μCi [^{35}S]methionine (~10 μl). The samples were incubated at 37°C in a 5% CO_2 incubator. After 4 h, medium was removed, the cells washed four times with PBS, and then harvested directly in 400 μl lysis buffer (9.5 M urea, 2% wt/vol NP-40, 1.6% ampholines pH range 5 to 7, 0.4% ampholines pH range 3 to 10, 5% β-mercaptoethanol). An aliquot was counted to determine the incorporation of [^{35}S]methionine into the fibroblast cells. The samples were then frozen in a dry ice-ethanol bath and stored at −20°C until required. The effect of exposure to DHT was determined by the addition of 50 nM DHT (in ethanol) for either 4 or 52 h (48 h before and 4 h during the labeling procedure). The same concentration of ethanol was added to control media.

Two-dimensional gel electrophoresis. Newly synthesized proteins in an aliquot of each sample (containing ~10^6 protein-bound cpm; mean±SD 975,000±66,000, $n = 10$) were resolved by two-dimensional gel electrophoresis exactly as described by O'Farrell (5). Spot intensities

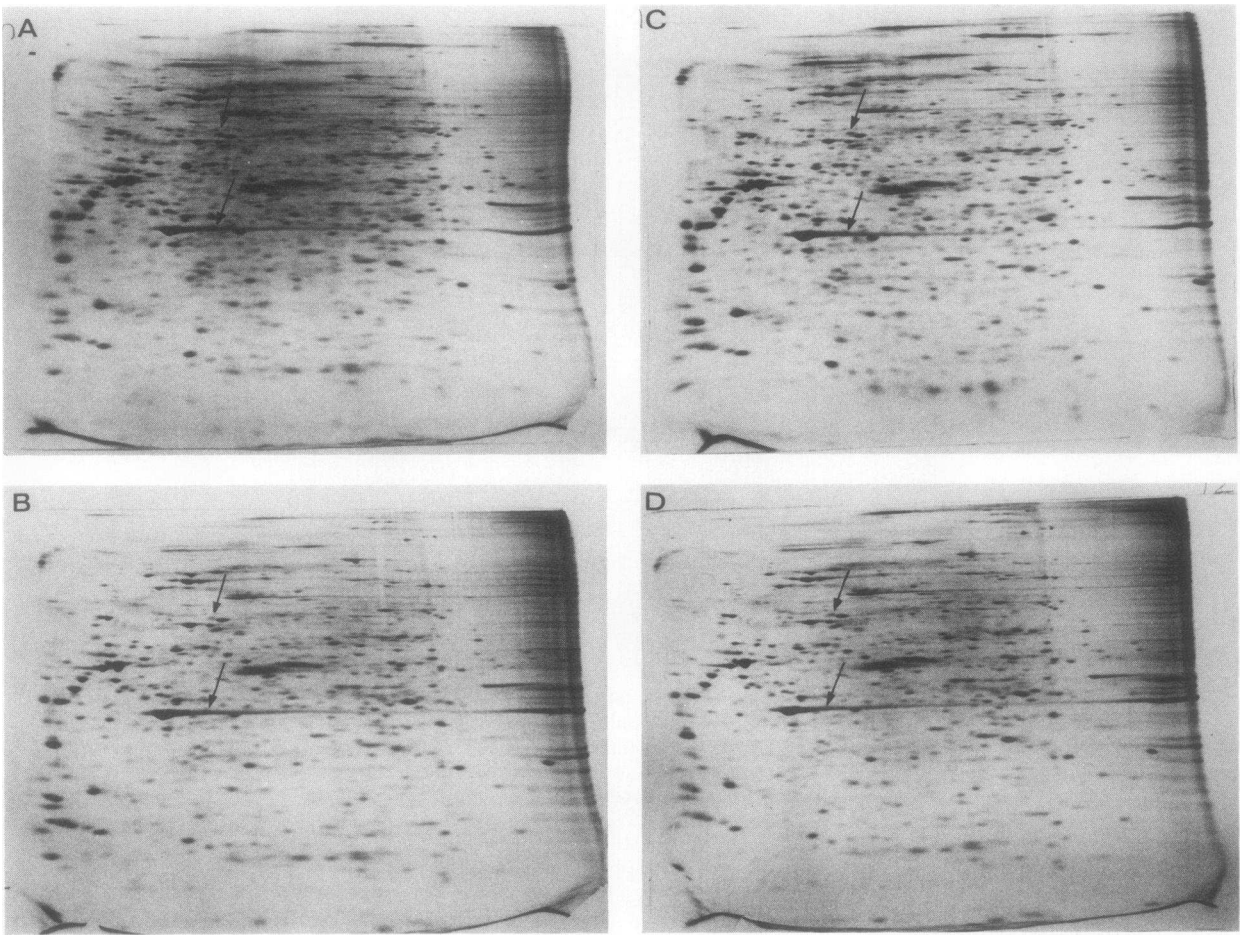

FIGURE 3 Pattern of protein synthesis by genital skin fibroblasts from a normal individual, NC13 (Fig. 3A, C) and a patient with testicular feminization, Te4 (Fig. 3B, D). The proteins were labeled with [^{35}S]methionine after exposure to DHT for 4 h (Fig. 3A, B) or for 52 h (Fig. 3C, D). Arrows indicate the position of two proteins that were more prominent in the gels from control fibroblasts compared with those from patients' fibroblasts.

were compared on gels exposed to film for the same period of time (48 h). Gels were compared by eye, and the differences between control and affected fibroblasts within individual runs recorded. Secondly, affected and control gels were examined between runs for differences within group. On this basis, consistent and reproducible variations between control and affected fibroblast protein synthetic profiles are described.

RESULTS

Androgen binding. The mean value for the binding of [^{3}H]R1881 to genital skin fibroblasts for normal males was 15.5±1.7 fm/10^6 cells (mean±SE), individual values ranging from 9.5 to 18.9 fm/10^6 cells. The average binding of [^{3}H]R1881 was genital skin fibroblasts from the three patients with testicular feminization was 1.5±0.3 fm/10^6 cells, a level ~10% that of normal.

Pattern of proteins synthesized by fibroblasts from normal individuals and those with testicular feminization. Fig. 1 shows the pattern of proteins synthesized by fibroblast cells from a normal individual (NC 13), with evidence of two proteins markedly more prominent than in cells from a patient with testicular feminization (Te4). The molecular weight of these proteins is estimated to be 85 and 45K; both proteins have a pK_i of ~5.0. These results were confirmed in two further experiments using fibroblast cells from two different normal and two different testicular feminized individuals: (NC 12 vs. Tela, NC 14 vs. Te3a); details of the gel region for the four cell lines are shown in Fig. 2.

The gels presented in Fig. 3 were simultaneously run with those in Fig. 1, and illustrate that the differences in protein synthesis were unaltered by ex-

posure to 50 nM DHT, in serum-free media, for 4 or 52 h.

DISCUSSION

These results demonstrate that there are two major differences between the patterns of proteins synthesized by fibroblast cells from normal individuals and patients with complete testicular feminization. The autoradiographs of the gels from control fibroblast cells showed two proteins of mol wt ~ 85 and ~ 45K, and pK_i ~ 5.0, consistently more prominent than in cells from patients with testicular feminization. These differences were reproducibly found in three different and unrelated patients with complete testicular feminization.

Mills and Bardin (8) have separated four androgen-induced proteins in mouse kidney preparations by single-dimension polyacrylamide gel electrophoresis. Additional studies with androgen-insensitive Tfm/Y mice indicated that stimulation of the proteins was dependent on a functional androgen receptor. In the present study, patterns of protein synthesis were unaltered by exposure to DHT at a concentration of 50 nM for either 4 or 52 h. These results suggest that there are differences in constitutive proteins of fibroblasts from normal individuals and those with complete testicular feminization. An alternative explanation is that of androgen-induced mRNA with such a long half-life that even after 52 h in androgen-free media no effects of androgen withdrawal on protein synthesis are discernible.

The molecular weight of the cytoplasmic androgen receptor in the prostate and epididymis is reported to be >200K (9, 10) with a pK_i of 5.8 (9). Additionally, the androgen receptor is known to be an oligomeric structure that dissociates under conditions of high ionic strength (11). It is therefore possible that the 85- and 45-K proteins represent a part of the androgen receptor complex, the levels of which are <10% of those in normal fibroblasts. The possibility that the 85- and 45-K proteins are subunits of the androgen receptor is open to investigation in several ways, including analysis of proteins synthesized by fibroblasts from those more unusual patients, who show androgen insensitivity despite apparently normal receptor levels assessed by androgen binding in vitro (4). Studies addressed to this possibility are currently in progress.

In conclusion, this study demonstrates a deficiency in synthesis of two proteins in genital skin fibroblasts obtained from patients with complete testicular feminization. The relationship between these proteins, the level of androgen receptors, and insensitivity to androgens remains to be elucidated.

ACKNOWLEDGMENTS

The excellent technical assistance of Miss Susan Pretty is gratefully acknowledged.

This work was supported by the National Health and Medical Research Council of Australia, the Anti-Cancer Council of Victoria, and by the Royal Children's Hospital Research Foundation.

REFERENCES

1. Grumbach, M. M., and J. S. Van Wyk. 1974. Disorder of sex differentiation. *In* Textbook of Endocrinology. R. H. Williams, editor. W. B. Saunders Company, Philadelphia, Pa. 5th edition. 423–501.
2. Morris, J. M., and V. B. Mahesh. 1963. Further observations on the syndrome, "testicular feminization." *Am. J. Obstet. Gynecol.* **87:** 731–734.
3. Kennan, B. S., W. J. Meyer III, A. J. Hadjian, H. W. Jones, and C. J. Migeon. 1974. Syndrome of androgen insensitivity in man: absence of 5α-dihydrotestosterone binding protein in skin fibroblasts. *J. Clin. Endocrinol. Metab.* **38:** 1143–1146.
4. Amrhein, J. A., W. J. Meyer III, H. W. Jones, Jr., and C. J. Migeon. 1976. Androgen insensitivity in man: evidence for genetic heterogeneity. *Proc. Natl. Acad. Sci. U. S. A.* **73:** 891–894.
5. O'Farrell, P. H. 1975. High resolution two-dimensional gel electrophoresis of proteins. *J. Biol. Chem.* **250:** 4007–4021.
6. Choo, K. H., R. G. H. Cotton, and D. M. Danks. 1976. Phenylalanine hydroxylation and tyrosine requirement of cultured cells. *Exp. Cell Res.* **101:** 370–382.
7. Griffin, J. E., K. Punyashthiti, and J. D. Wilson. 1976. Dihydrotestosterone binding by cultured human fibroblasts. Comparison of cells from control subjects and from patients with hereditary male pseudohermaphroditism due to androgen resistance. *J. Clin. Invest.* **57:** 1342–1351.
8. Mills, N. C., and C. W. Bardin. 1980. New androgen-stimulated proteins in the kidneys of female mice. *Endocrinology.* **106:** 1182–1186.
9. Mainwaring, W. I. P. 1969. A soluble androgen receptor in the cytoplasm of rat prostate. *J. Endocrinol.* **45:** 531–541.
10. Hansson, V., S. C. Weddington, F. S. French, W. McLean, A. Smith, S. N. Nayfeh, E. M. Ritzen, and L. Hagenas. 1976. Secretion and role of androgen-binding proteins in the testis and epididymis. *J. Reprod. Fertil.* **24**(Suppl): 17–33.
11. Baulieu, E-E., and I. Jung. 1970. A prostatic cytosol receptor. *Biochem. Biophys. Res. Commun.* **38:** 599–606.