THE LANCET

Vol 337 Saturday 11 May 1991 No 8750

ORIGINAL ARTICLES

Organ-specific cardiac antibodies: serological markers for systemic hypertension in autoimmune polyendocrinopathy

ALIDA L. P. CAFORIO RICHARD WAGNER JASWINDER S. GILL
EZIO BONIFACIO EMANUELE BOSI AMANDA MILES
WILLIAM J. MCKENNA GIAN FRANCO BOTTAZZO

Circulating organ-specific autoantibodies are serological markers of destruction or impairment of the relevant endocrine tissue cells and may be associated with abnormal hormone levels with or without clinical evidence of overt disease. We sought organ-specific cardiac antibodies in patients with autoimmune polyendocrinopathy because of increasing evidence that the heart has endocrine characteristics (secretion of atrial natriuretic peptide [ANP] and other peptide hormones). Serum samples from 166 patients with polyendocrinopathy, 80 with autoimmunity confined to one gland, and 200 healthy blood donors were tested for these antibodies by means of immunofluorescence on human heart. Skeletal muscle was used to identify cross-reacting antibodies. Organ-specific cardiac antibodies were detected in significantly more of the patients with autoimmune polyendocrinopathy (28 [17%]) than of those with autoimmunity confined to one gland (1 [1%]) or of normal subjects (7 [3·5%]; $p = 0·0001$). Among the patients with autoimmune polyendocrinopathy, the prevalence of systemic hypertension was higher in those with cardiac autoantibodies than in those without (5/28 [18%] *vs* 2/80 [3%]; $p = 0·01$); the same was true for a family history of hypertension (11 [42%] *vs* 5 [7%]; $p = 0·0001$). There were no significant differences in mean basal or stimulated ANP concentrations between patients with or without antibodies or between patients and controls. 5 of the 22 antibody-positive patients had ANP concentrations outside the normal range, but these disturbances were not associated with systemic hypertension or a family history of the disorder. Patients with autoimmune polyendocrinopathy can have organ-specific cardiac antibodies, which may represent novel serological markers for an autoimmune form of systemic hypertension in the absence of overt cardiac disease.

Lancet 1991; **337**: 1111–15.

Introduction

Organ-specific autoimmunity largely affects endocrine organs and, in genetically predisposed individuals, autoimmune endocrine disorders tend to group together.[1-4] Patients with autoimmune polyendocrinopathy have many organ-specific autoantibodies, which are serological markers of damage or destruction of the corresponding endocrine cells. Their detection may precede the onset of overt disease by several years or be associated with abnormal hormone levels with or without apparent clinical manifestations.[1,2]

The heart seems to have endocrine-like functions—there is active synthesis and secretion of atrial natriuretic peptide (ANP), primarily by atrial myocytes.[5] We have found "organ-specific" cardiac autoantibodies in patients with

ADDRESSES: **Department of Cardiological Sciences, St George's Hospital Medical School** (A. L. P. Caforio, MD, J. S. Gill, MRCP, W. J. McKenna, MD); **Department of Immunology, University College and Middlesex School of Medicine** (A. L. P. Caforio, E. Bonifacio, PhD, Prof G. F. Bottazzo, FRCP); **Department of Diabetes and Immunogenetics, St Bartholomew's Hospital, London, UK** (R. Wagner, MD,* A. Miles, RGN, Prof G. F. Bottazzo); **and San Raffaele Hospital Scientific Institute, Milan, Italy** (E. Bosi, MD). *Present address: Department of Endocrinology, Medizinische Klinik, Essen, Germany. Correspondence to Prof G. F. Bottazzo, London Hospital Medical College, Department of Immunology, 56 Ashfield Street, London E1 2AD, UK.

dilated cardiomyopathy, a disorder suspected to be autoimmune and characterised by chronic heart failure.[6]

The detection of "organ-specific" cardiac antibodies in polyendocrinopathic patients without overt heart dysfunction might suggest a role for these antibodies as early markers of cardiovascular disease. We have studied the prevalence of organ-specific cardiac antibodies in a group of such patients, in patients with only one autoimmune endocrinopathy, and in normal subjects. We carried out detailed cardiovascular assessment to search for subclinical heart dysfunction and/or positive family history of cardiovascular disease in the polyendocrinopathic patients. Plasma concentrations of the putative cardiac hormone, ANP, were also measured.

Patients and methods

Serum samples were obtained from 166 patients with polyendocrine autoimmune disease, 53 with autoimmune thyroid disease only, and 27 with insulin-dependent diabetes mellitus (IDDM) of recent onset, apparently not complicated by other endocrine abnormalities. Samples from 200 blood donors served as controls for the indirect immunofluorescence studies.

The mean (SD) age of the autoimmune polyendocrinopathy patients was 45 (15) years; the 115 women and 51 men all lived in southern England. Their serum samples were tested at the autoimmune serology laboratory at the Middlesex Hospital, London, between Jan 1, 1976, and Jan 1, 1987, and various organ-specific autoantibodies were detected. Detailed personal and family histories, as well as clinical information, were initially obtained from a questionnaire completed by their doctors (general practitioners or consultants). Clinical classification of endocrine/organ-specific disease was based on the diagnosis given by the referring physician. All 166 polyendocrine patients had at least two glands and/or tissues (ie, stomach) affected clinically by organ-specific autoimmune disease and/or had circulating autoantibodies to the relevant organ.[7]

The distribution of the clinical and serological autoimmune manifestations in different organs among these patients is shown in fig 1. The autoimmune diseases affecting these patients included thyrotoxicosis (in 28), primary myxoedema (41), Hashimoto's thyroiditis (11), pernicious anaemia (18), IDDM (56), Addison's disease (19), idiopathic amenorrhoea (2), and vitiligo (15). Organ-specific antibodies included antibodies to islet cell (142), gastric parietal cell (116), pituitary (4), adrenal (40), thyroglobulin (75), and thyroid microsomes (130). 23% of these patients were positive for at least two autoantibodies but did not have any clinically overt autoimmune disease (fig 1).

The mean age of the 51 women and 2 men with clinical and/or serological autoimmune thyroid disease was 45 (18) years. 5 had thyroid antibodies but no clinical disease, 16 had thyrotoxicosis, 22 primary myxoedema, and 10 Hashimoto's thyroiditis. All had antibodies to thyroglobulin (51) and/or thyroid microsomes (53). 15 patients also had gastric parietal cell antibodies. The patients with recent-onset IDDM had a mean age of 21 (9) years; they were all male. They entered the study within 1 year of diagnosis; 21 had islet cell antibodies. The 200 healthy blood donors had a mean age of 35 (11) years; there were 109 women and 91 men.

For detection of cardiac autoantibodies serum samples were initially tested by indirect immunofluorescence at 1/10 dilution on 4 μm unfixed fresh frozen cryostat sections of blood group O normal human atrium, ventricle, and skeletal muscle.[6] All positive samples were titrated to endpoint on cardiac sections. As internal control for assay detection limit, two samples were chosen as standard positive and negative controls and titrated in every assay. The intensity of immunofluorescence of the positive standard at 1/40 dilution was used as the threshold of positivity. All samples tested at 1/10 dilution were read against these standards by two independent observers unaware of the clinical details. An additional control serum was tested to assess reproducibility.[6] Endpoint titres for this serum were between 1/40 and 1/80 in 14 of 15 assays.

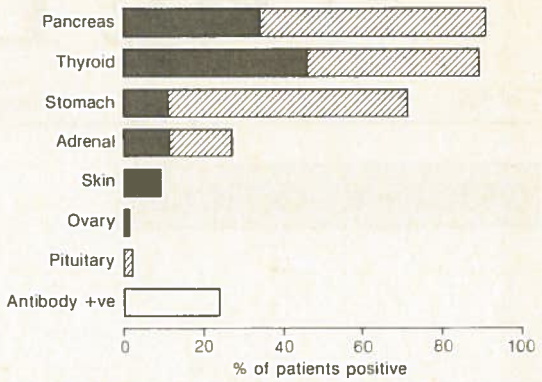

Fig 1—Distribution of clinical and serological organ-specific autoimmune manifestations in polyendocrinopathic patients.

■ overt clinical disease, with or without positive serology; ▨ associated organ-specific antibody, no clinical disease; □ organ-specific autoimmune serology only (at least two organs).

For the detection of other organ-specific antibodies standard indirect immunofluorescence methods and haemagglutination assay (for thyroid microsomal and thyroglobulin antibodies) were used.[7]

We contacted all 166 polyendocrinopathic patients included in the cardiac autoantibody screening. The 28 patients with organ-specific cardiac antibodies and 80 of the 118 without these antibodies consented to undergo detailed medical history and clinical evaluation by at least two of us and were recruited for the clinical association study. The cardiovascular assessment included: personal and family history of cardiovascular disease, including systemic hypertension (blood pressure above 150/90 mm Hg), coronary artery disease symptoms, and family history of these disorders in first and second degree relatives; clinical evaluation with blood pressure measurement; 12 lead electrocardiogram; chest radiograph and/or two-dimensional echocardiographic and doppler evaluation by conventional protocols.

Basal (after 10 min standing) and stimulated (after 20 min supine with leg-raising) plasma ANP concentrations were measured by standard radioimmunoassay[8] in 22 consecutive patients with organ-specific cardiac antibody, 20 antibody-negative polyendocrinopathic patients matched for age and disease, and 22 healthy controls matched for age and sex. Blood was collected and centrifuged immediately at 4°C; plasma was stored at −20°C. ANP was extracted by passage of 3 ml plasma through a preactivated 'Sep-Pak C18' cartridge (Waters Associates, Milford, Massachusetts, USA) and eluted with a mixture of acetonitrile and water (60/40 by volume). The solvent was evaporated in a centrifugal drier. The ANP was resuspended in 1 ml assay buffer and assayed with ANP antibody (Peninsula Laboratories, St Helens, UK) and ANP tracer (Amersham, Aylesbury, UK).

The chi-square test or Fisher's exact test was used to compare the prevalence of cardiac antibodies in disease and normal groups and to assess the association of clinical features with serological findings as well as the association of clinical diagnoses with systemic hypertension and positive family history of this disorder. The unpaired Student's *t* test was used to compare mean basal and stimulated ANP concentrations among the study groups.

TABLE I—PREVALENCE OF CARDIAC ANTIBODIES IN ENDOCRINE AUTOIMMUNE DISEASE

	No (%)		
	Organ-specific	Cross-reactive 1	Cross-reactive 2
Polyendocrine autoimmune disease (n=166)	28* (17%)	10 (6%)	10 (6%)
Autoimmune thyroid disease (n=53)	1 (2%)	1 (2%)	2 (4%)
Newly diagnosed IDDM (n=27)	0	0	0
Blood donors (n=200)	7 (3·5%)	3 (1·5%)	8 (4%)

*Chi-square test for comparison with normal subjects, p=0·0001

borderline basal ANP concentration. Among antibody-negative patients and normals there was no paradoxical ANP response to stimulation.

No significant association was found between the presence of abnormal ANP concentrations and any particular clinical diagnosis, including systemic hypertension; ANP abnormalities were detected in similar proportions of patients with and without hypertension (1/5 [20%] *vs* 4/17 [23%]) and of patients with and without a positive family history (1/9 [11%] *vs* 4/13 [31%]).

Discussion

Patients with autoimmune polyendocrinopathy have been invaluable for the identification of novel autoantibody specificities.[9-12] Autoimmune polyendocrinopathy was lately associated with Stiff-man syndrome[13] and it was within this association that autoantibodies to GABA-ergic neurons[14] and to glutamic acid decarboxylase, also one of the putative islet autoantigens in IDDM,[15] were first identified. Our finding of organ-specific cardiac antibodies in dilated cardiomyopathy[6] and the suggested endocrine function of myocytes by their synthesis and secretion of peptides (eg, ANP),[5] led us to postulate that myocytes might be liable to an autoimmune attack similar to that exerted against other endocrine organs. The detection of cardiac antibodies with organ-specific reactivity in serum from patients with autoimmune polyendocrinopathy is as we expected.

Organ-specific antibodies are serological markers of a clinical or subclinical dysfunction of the corresponding target organ. Organ-specific cardiac antibodies are associated with dilated cardiomyopathy,[6] but these antibodies were found in autoimmune polyendocrinopathy in the absence of overt cardiac dysfunction. In the polyendocrinopathic patients, however, there was a positive association with systemic hypertension and with a family history of hypertension. Systemic hypertension is not found in dilated cardiomyopathy, which suggests that the clinical relation of these autoantibodies in autoimmune polyendocrinopathy differs from that in dilated cardiomyopathy. The immunofluorescent staining patterns of cardiac antibodies in dilated cardiomyopathy and in autoimmune polyendocrinopathy are similar, but recognition by distinct antibodies of various cardiac autoantigens may account for the differences in the observed clinical associations. Potential cardiac-specific cytoplasmic autoantigens include myosin isoforms,[16-18] cardiac isoforms of other contractile proteins,[17,18] and mitochondrial[19,20] and microsomal antigens.[21,22]

Systemic hypertension is a complex disorder and, in most cases, its aetiology is unknown. The possibility that the high blood pressure in the autoimmune polyendocrinopathic patients with organ-specific cardiac antibodies was due to poor hormonal control[23,24] was excluded by the finding that all of them had hormone concentrations within the normal ranges. Furthermore, the finding of systemic hypertension was not associated with any particular autoimmune disease. The concomitant association of these antibodies with a family history of coronary artery disease is probably related to systemic hypertension, the latter being an important risk factor for development of coronary artery disease.[25] Organ-specific cardiac antibodies, similarly to other autoantibody specificities, might precede overt clinical manifestations of disease. In 1 of our polyendocrinopathic patients (patient 22), these antibodies were detected a year before systemic hypertension developed. Follow-up of the patients who are cardiac antibody positive but without hypertension will allow us to assess the potential role of these new antibodies in prediction of disease onset.

We suggest that the presence of organ-specific cardiac antibodies in polyendocrinopathy may characterise an autoimmune form of systemic hypertension. We suspected abnormalities of ANP secretion as a possible factor to explain this association. In our study, however, there was no significant association between the postulated defect of ANP secretion and hypertension.

The physiological role of ANP in blood pressure regulation is controversial.[26] Plasma ANP within the normal range has been reported in patients with mild essential hypertension, but it is raised in moderate to severe hypertension in association with left ventricular hypertrophy and/or heart or renal failure; this might represent a compensatory effect due to increased intracardiac pressures and/or hypervolaemia.[26] These data, and experimental data,[27] do not support a key role for the cardiac hormone as a natriuretic substance in the long-term regulation of blood pressure. Our results in polyendocrinopathic patients with organ-specific cardiac antibodies and systemic hypertension are consistent with this view. Alternatively, the part played by ANP may be small and overwhelmed by the effect of other cardiac peptides, such as iso-rANP or brain natriutretic peptide.[28,29] Longitudinal studies are needed to see whether the ANP abnormalities detected in a minority of our patients with cardiac antibodies (fig 2) will be associated with and ultimately lead to any precise clinical syndrome. Most importantly, circulating basal and stimulated levels of the newly identified cardiac peptides should also be measured.

Prospective studies are now needed to confirm whether these antibodies characterise a subtype of autoimmune systemic hypertension, and to see if their detection may be useful in the identification of patients at risk of systemic hypertension or other cardiovascular disease.

We thank all the patients who collaborated in the Polyendocrine Study and the referring physicians for their continuing interest; Ms P. Collins and Ms M. Patel for the initial antibody screen; Dr J. Poloniecki for help in the statistical analysis; and Mrs Kathy Cusack for typing the paper. This study was supported by the Juvenile Diabetes Foundation International (JDF-I), USA. A. L. P. C. is supported by the British Heart Foundation, R. W. was granted a Travelling Fellowship from Henning Pharmaceutical, Berlin, and the B-Braum Stiftung Nelsungen, Germany, and E. Bonifacio is a research fellow of the JDF-I.

REFERENCES

1. Bottazzo GF, Todd I, Mirakian R, Belfiore A, Pujol-Borrell R. Organ-specific autoimmunity. A 1986 overview. *Immunol Rev* 1986; **94**: 137–69.
2. Bottazzo GF, Doniach D. Polyendocrine autoimmunity: an extended concept. In: Volpe R, ed. Autoimmunity and endocrine disease. New York: Marcel Dekker, 1985: 37–49.
3. Claude H, Gougerot H. Insuffisance pluriglandulaire endocrienne. *J Physiol Pathol Gen* 1908; **10**: 469–80.
4. Neufeld M, Blizzard RM. Polyglandular autoimmune disease. In: Pinchera A, Doniach D, Fenzi GF, Baschieri L, eds. New York: Academic Press, 1980: 357–565.
5. Goetz KL. Physiology and pathophysiology of atrial peptides. *Am J Physiol* 1988; **254**: E1–15.
6. Caforio ALP, Bonifacio E, Stewart JT, et al. Novel organ-specific circulating cardiac autoantibodies in dilated cardiomyopathy. *JACC* 1990; **15**: 1527–34.
7. Bosi E, Becker F, Bonifacio E, et al. Progression to type 1 (insulin-dependent) diabetes in autoimmune endocrine patients with islet cell antibodies. *Diabetes* (in press).
8. Donckier J, Anderson JV, Bloom SR. Alpha atrial natriuretic peptide concentrations in plasma in children with congenital heart and pulmonary diseases. *Br Med J* 1985; **291**: 1648–49.

Results

As in our previous study[6] of dilated cardiomyopathy, we detected cardiac antibodies with three distinct immunofluorescence staining patterns: organ-specific antibodies, which stained diffusely the cytoplasm of atrial and, to a lesser extent, ventricular myocytes; cross-reactive 1 antibodies, which gave fine striational staining on cardiac tissue, but only weak staining on skeletal muscle fibres; and crossreactive 2 antibodies, which stained with a broad striational pattern both heart and skeletal muscle sections. Absorption studies with relevant tissues confirmed the organ-specificity and cross-reactivity of the three antibody types.

Organ-specific cardiac antibodies were detected in significantly more of the patients with polyendocrine autoimmune disease than of the healthy blood donors (table I). Skeletal muscle crossreactive 1 and 2 antibodies were found in similar proportions among patients and normal subjects (table I).

The titres of both organ-specific and crossreactive cardiac antibodies ranged from 1/10 to 1/40. The antibodies were all IgG. 4 samples contained also IgM and 2 IgA in addition to IgG.

Table II lists the endocrine disorders in the 28 patients with organ-specific cardiac antibodies. All patients were clinically well controlled with or without the appropriate hormone replacement therapy and had hormone concentrations within the normal ranges. The finding of organ-specific cardiac antibody was not significantly associated with age, with any particular autoimmune disease, or the presence of other organ-specific autoantibodies.

Of the 28 patients with organ-specific cardiac antibody, all but 1 (patient 25) were without clinical, radiographic, and/or echocardiographic evidence of left or right heart failure or ventricular wall hypertrophy (table II). 5 of 28 patients had systemic hypertension but their blood pressure was well controlled by treatment and they were normotensive at the time of cardiac examination and of blood sampling for ANP. In 2 of these 5 patients systemic hypertension was associated with IHD (table II). None of the 80 cardiac-antibody-negative polyendocrinopathic patients examined had clinical or radiographic evidence of left or right ventricular dysfunction, but 2 of them were receiving treeatment for systemic hypertension. The prevalence of systemic hypertension was significantly higher among patients with organ-specific cardiac antibodies than in those without such antibodies (5/28 [18%] *vs* 2/80 [3%], $p = 0{\cdot}01$). The prevalence of IHD did not differ significantly between these subgroups (2 [7%] *vs* 3 [4%]).

A complete family history for cardiovascular disease was obtained in 26 of the 28 polyendocrine patients with organ-specific cardiac antibodies and in 67 of the 80 who were antibody negative. The presence of organ-specific cardiac antibodies was associated with a family history of hypertension (11 [42%] *vs* 5 [7%], $p = 0{\cdot}0001$) and family history of IHD (8 [31%] *vs* 6 [9%] $p = 0{\cdot}01$). The presence of systemic hypertension or positive family history was not associated with any particular autoimmune disease among the patients who underwent cardiovascular assessment.

Basal ANP levels did not differ significantly between the group with organ-specific cardiac antibodies (5·8 [5·8] pmol/l) and the antibody-negative group (4·8 [1·1] pmol/l) or the healthy controls (4·6 [1·2] pmol/l). Stimulated ANP levels were also similar within the three study groups (10·5 [4·5]; 10·0 [2·0]; 9·6 [2·3] pmol/l, respectively). However, 5 of 22 patients with cardiac antibodies had values above or below the range of values in the normal group (fig 2). Patient 25, who had hypertension and congestive heart failure, had high basal and stimulated ANP concentrations. In the other 4 patients with abnormal basal and/or stimulated ANP concentrations there was no apparent clinical association. Only 1 antibody-negative control patient had a low

TABLE II—CLINICAL DATA ON POLYENDOCRINE PATIENTS WITH CARDIAC SPECIFIC ANTIBODY

Patient	Age (yr)	Sex	Clinical disorders	Family history: Hyper-tension	Family history: IHD
1	35	F	None	+	−
2	37	F	Thyrotoxicosis	−	−
3	66	F	Primary myxoedema, hypertension	+	+
4	66	F	IDDM, thyrotoxicosis, pernicious anaemia, chronic active hepatitis	−	+
5	36	M	IDDM	−	−
6	54	F	Primary myxoedema	−	−
7	28	F	Addison's, hypoparathyroidism	−	+
8	49	F	IDDM	+	−
9	25	M	Addison's	+	+
10	55	M	Vitiligo, hypertension, IHD	−	+
11	40	F	IDDM, Addison's, thyrotoxicosis	−	−
12	48	F	Addison's, thyrotoxicosis	−	−
13	56	F	IDDM	?	?
14	44	F	None	−	−
15	38	F	IDDM	−	−
16	61	F	Hashimoto's thyroiditis	+	−
17	40	F	Primary myxoedema	+	+
18	62	M	IDDM, euthyroid post-thyrotoxicosis	−	−
19	37	F	Primary myxoedema	+	+
20	25	F	IDDM, polycystic ovaries	−	+
21	28	M	IDDM	?	?
22	47	F	Hashimoto's thyroiditis, hypertension	+	−
23	22	F	IDDM, primary myxoedema	−	−
24	50	F	Euthyroid post-thyrotoxicosis, polycystic ovaries	−	−
25	52	F	IDDM, Hashimoto's thyroiditis, vitiligo, hypertension, IHD	−	−
26	68	F	IDDM, vitiligo, hypertension	+	−
27	41	M	IDDM, Addison's, primary myxoedema, vitiligo	+	−
28	45	M	Type 2 DM, primary myxoedema	+	−

IHD = ischaemic heart disease

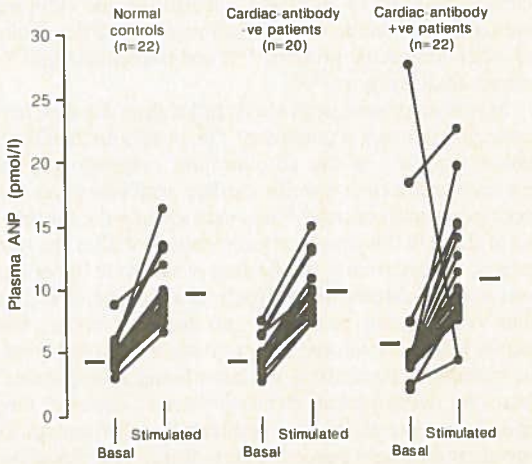

Fig 2—ANP response in patients with and without cardiac antibodies and normal subjects.

Bars = mean values.

9. Bottazzo GF, Florin-Christensen A, Doniach D. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. *Lancet* 1974; ii: 1279–83.
10. Bottazzo GF, Pouplard A, Florin-Christensen A, Doniach D. Autoantibodies to prolactin-secreting cells of human pituitary. *Lancet* 1975; ii: 97-101.
11. Scherbaum WA, Bottazzo GF. Autoantibodies to vasopressin-cells in idiopathic diabetes insipidus: evidence for an autoimmune variant. *Lancet* 1983; i: 897–901.
12. Mirakian R, Richardson AC, Milla AC, et al. Protracted diarrhoea of infancy: evidence in support of an autoimmune variant. *Br Med J* 1986; **293:** 1132–36.
13. Bosi E, Vicari A, Comi G, et al. Association of Stiff-Man syndrome and type 1 diabetes with islet cell and other autoantibodies. *Arch Neurol* 1988; **54:** 246–47.
14. Solimena M, Folli F, Aparisi R, Pozza G, De Camilli P. Autoantibodies to GABA-ergic neurons and pancreatic beta cells in Stiff-Man syndrome. *N Engl J Med* 1990; **322:** 1555–60.
15. Baekkeskov S, Aanstoot HJ, Christgau S, et al. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxilase. *Nature* 1990; **347:** 151–56.
16. Neu N, Rose NR, Beisel KW, Herskowitz A, Gurri-Glass G, Craig SW. Cardiac myosin induces myocarditis in genetically predisposed mice. *J Immunol* 1987; **139:** 3630–36.
17. Swynghedauw B. Developmental and functional adaptation of contractile proteins in cardiac and skeletal muscles. *Physiol Rev* 1986; **66:** 710–71.
18. Nadal-Girard B, Mahdavi V. Molecular basis of cardiac performance. Plasticity of the myocardium generated through protein isoform switches. *J Clin Invest* 1989; **84:** 1693–700.
19. Klein R, Maisch B, Kochsiek K, Berg PA. Demonstration of organ-specific antibodies against heart mitochondria (anti-M7) in sera from patients with some forms of heart disease. *Clin Exp Immunol* 1979; **58:** 283–92.
20. Schulteiss HP, Bolte HD. Immunological analysis of autoantibodies against the adenine nucleotide transolactor in dilated cardiomyopathy. *J Mol Cell Cardiol* 1985; **17:** 603–17.
21. Kaplan MH, Meyeseran M. Immunologic studies of heart tissue. V Antigens related to heart tissue revealed by cross-reaction of rabbit antisera to heterologous heart. *J Immunol* 1962; **88:** 450–61.
22. Espinosa E, Kushner I, Kaplan MH. Antigenic composition of heart tissue. *Am J Cardiol* 1969; **24:** 508–13.
23. Streeten DHP, Anderson GH, Howland T, Chiang R, Smulyan H. Effects of thyroid function on blood pressure. Recognition of hypothyroid hypertension. *Hypertension* 1988; **11:** 78–83.
24. Trevisan R, Fioretto P, Semplicini A, et al. Role of insulin and atrial natriuretic peptide in sodium retention in insulin-treated IDDM patients during isotonic volume expansion. *Diabetes* 1990; **39:** 289–98.
25. Kannel WB. Role of blood pressure in cardiovascular disease: the Framingham Study. *Angiology* 1975; **26:** 1–14.
26. Genest J, Larochelle P, Cusson JR, Gutkowska J, Cantin M. The atrial natriuretic factor in hypertension. State of the art lecture. *Hypertension* 1988; 11 (suppl I): 13–17.
27. Greenwald JE, Sakata M, Michener ML, Sides SD, Needleman P. Is atriopeptin a physiological or pathophysiological substance? Studies in the autoimmune rat. *J Clin Invest* 1988; **81:** 1036–41.
28. Jennings DB, Flynn TG. The disulfide bonded ring of iso-rANP, unlike that of rANP has potent cardiovascular activity. *Can J Physiol Pharmacol* 1990; **68:** 131–36.
29. Tateyama H, Hino J, Minamino N, Kangawa K, Ogihara T, Matsuo H. Characterization of immunoreactive brain natriuretic peptide in human cardiac atrium. *Biochem Biophys Res Commun* 1990; **166:** 1080–87.

Diagnosis of genetic disease by primer-specified restriction map modification, with application to cystic fibrosis and retinitis pigmentosa

ERIC J. SORSCHER ZHEN HUANG

Detection of small alterations or abnormalities in genomic DNA (eg, point mutations or small deletions) has become increasingly important in the diagnosis of genetic disease and polymorphism. When a mutation or polymorphism creates a new restriction endonuclease site, it can easily be identified by polymerase chain reaction (PCR) amplification of the DNA region of interest, followed by digestion with the restriction endonuclease. However, useful restriction sites are the exception, and a variety of specialised techniques have been developed to identify subtle DNA abnormalities. We have shown that where a DNA mutation does not create a useful novel restriction site, such a site can be introduced by PCR and specially chosen primers. The approach is simple and inexpensive and should be broadly applicable in the diagnosis of genetic polymorphism and mutation. The technique is illustrated here by the three base-pair deletion responsible for most cases of cystic fibrosis and by detection of the point mutation in the rhodopsin gene that has been associated with some cases of autosomal dominant retinitis pigmentosa.

Lancet 1991; **337:** 1115–18.

Introduction

A growing number of genetic diseases and other phenotypic polymorphisms can now be identified at the level of the responsible gene. Haemoglobinopathies, muscular dystrophy, cystic fibrosis, neurofibromatosis, and Lesch-Nyhan disease are examples of hereditary conditions that are amenable to DNA diagnosis,[1-5] and histocompatibility type is an illustration of a polymorphism that can be characterised in this way too.[3] The DNA alteration responsible is often just a single base change or small deletion or insertion, and several techniques have been devised to identify small lesions or variable regions of DNA. When a small change in DNA creates or destroys a unique restriction site, the presence or absence of a mutation can be identified in a sample of DNA simply by cutting the region of DNA of interest with a specific restriction enzyme. Examples are the β-globin mutation in sickle-cell anaemia and the defective allele in the factor VIII gene associated with haemophilia A. In mutations such as these, the DNA region of interest may be amplified by the polymerase chain reaction (PCR) before digestion with a restriction enzyme. This approach only works where a natural mutation causes a useful change in a restriction site, and that is rare. As a result, other techniques have evolved.

Allele-specific oligonucleotide hybridisation is the most widely used alternative approach, permitting even a single base change to be identified. Other approaches are competitive oligonucleotide priming PCR, ligase-mediated allele detection, multiplex PCR detection, cleavage

ADDRESS: **Department of Medicine, Division of Hematology-Oncology, and Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA** (E. J. Sorscher, MD, Z. Huang, MD). Correspondence to Dr Eric J. Sorscher.

mismatch detection, denaturing gradient gel electrophoresis, and identification of single-stranded DNA polymorphism—but all are more complex than simple PCR amplification followed by restriction digestion. Oligonucleotide specific hybridisation, for example, requires great care with hybridisation and washing conditions and radioactive probes or non-radioactive colorimetric techniques. Competitive oligonucleotide priming PCR[6,7] refers to detection of polymorphic alleles using the PCR and primers encompassing a polymorphic region of interest. Under carefully specified conditions, a PCR product will only be obtained if the primer exactly matches the template. However, PCR primers tend to amplify despite significant mismatch with template—and it is this observation that forms the basis of PCR-directed mutagenesis. No-one doubts that competitive PCR priming can offer rapid diagnosis of DNA polymorphism but technical difficulties have left allele-specific hybridisation as the procedure of choice for detecting small changes in DNA.

Restriction map modification

The approach used here is summarised in fig 1. The region of DNA under study is restriction mapped—ie, a computer print-out is obtained showing restriction enzyme recognition sites within the region of interest. If a unique restriction site is already present due to the polymorphic sequence itself, standard PCR and digestion can be used, provided there are no such sites elsewhere to complicate interpretation of the restriction digests. If no useful restriction site is present, one is introduced by primer-specified restriction map modification. A list of restriction endonuclease recognition sites is consulted to choose sites that more closely resemble the polymorphic allele under study than other alleles at the same position in the locus. Primers are chosen around the area of interest so that a mutation will be introduced into a PCR product. The mutation is designed so that it will create a new restriction site in a PCR product from the polymorphic allele of interest but not in products from the wild type allele or from other polymorphic alleles which are not being targeted.

Applications to cystic fibrosis ΔF_{508} deletion

In cystic fibrosis (CF), the most common defective allele has a deletion of three base-pairs (CTT) in the CF gene and is known as ΔF_{508}. The enzyme *Cla*I recognises the sequence 5'ATCGAT3'. This sequence is not present in either the defective gene (CF) or the wild type (normal) allele but it can be introduced if two bases of the CF allele are changed (fig 2). Amplification of the wild type allele also leads to a PCR product but since this allele has three extra base pairs in the polymorphic region, a new *Cla*I site should not be introduced. The predicted sequence of the PCR product would be 5'AAAGAAAATATCATCTTCGATGTT3': the *Cla*I site would thus be destroyed by the extra bases in the wild type allele and only the mutant allele would be predicted to be cut with this enzyme.

Application to autosomal dominant retinitis pigmentosa

Some cases of autosomal dominant retinitis pigmentosa have been attributed to a point mutation in the rhodopsin gene.[2,8] The normal sequence in the region of the point mutation and the sequence of the mutated allele are shown in fig 3. The point mutation is a C→A transversion. The enzyme *Dra*III should be able to identify this mutation after

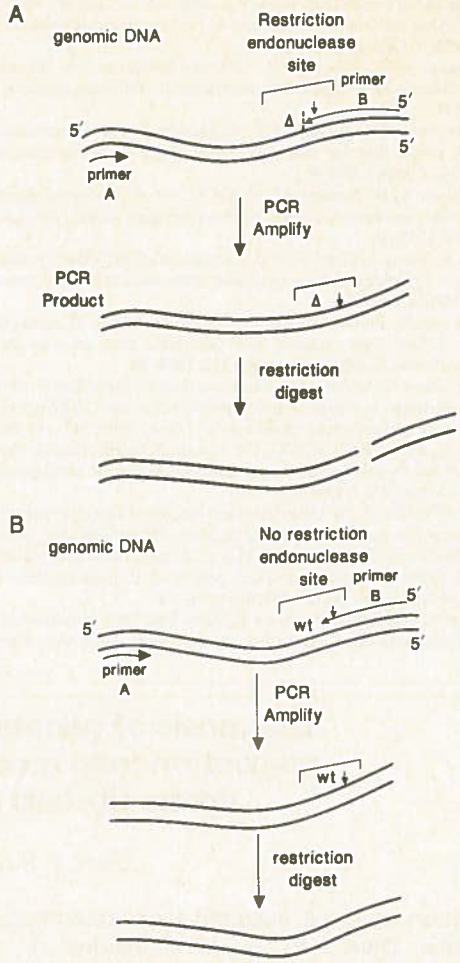

Fig 1—Restriction map modification using PCR.

Region surrounding polymorphism or mutation of interest (△) is amplified by PCR. One amplification primer is chosen to introduce new mutation (small arrow) in the PCR product. This mutation creates a novel restriction endonuclease site in PCR product from polymorphic allele of interest (A) but not from wild type allele (B).

PCR amplification, since it recognises the sequence 5'CACNNNGTG3', where N is any base. A *Dra*III site would be expected in a PCR product for the mutant, but not the normal allele, if an A→T mutation is placed in the position shown by the asterisk in fig 3.

Methods

PCR and endonuclease cutting

Whole blood was used to prepare PCR-suitable genomic DNA.[9] Briefly, 0·5 ml blood was added to 0·8 ml lysis buffer (0·32 mol/l sucrose, 10 mmol/l "tris" HCl pH 7·5, 5 mmol/l magnesium chloride, and 1% 'Triton X-100') in a 1·5 ml tube. Samples were spun at 13 000 *g* for 20 s, supernatants were discarded, and pellets resuspended in 1·0 ml lysis buffer; centrifugation and resuspension steps were repeated twice more. After a final spin (13 000 *g*, 20 s) pellets were resuspended in 500 µl buffer containing 50 mmol/l KCl, 10 mmol/l tris HCl pH 8·3, 2·5 mmol/l magnesium chloride, 0·1 mg/ml gelatin, 0·45% NP40, and 0·45% 'Tween 20'. 0·6 µl of a 10 mg/ml solution of proteinase K in water was added to every 100 µl of buffer just before use. Samples were incubated at 50–60°C for 1 h and then at 90°C for 10 min to inactive proteinase K, and then stored frozen. Tissue samples and cell-culture material were also used for DNA preparation for PCR.[9,10]