petection of Mycobacterium Tuberculosis DNA in Fresh Skin Samples of Various Granulomatous Skin Diseases by Polymerase Chain Reaction: a preliminary report

ÇEŞİTLİ GRANULOMATOZ DERİ HASTALIKLARINA AİT TAZE DERİ BİYOPSİ ÖRNEKLERİNDE POLİMERAZ ZİNCİR REAKSİYONU YÖNTEMİ İLE MYCOBACTERIUM TUBERCULOSIS DNA'SININ SAPTANMASI: bir ön çalışma

Nilgün BİLEN*, Rebiay APAYDIN*, C. ERCİN**, Yeşim GÜRBÜZ**, Dilek BAYRAMGÜRLER*, H. VAHABOĞLU***

Depts. of * Dermatology, **Pathology and, ***Clinical Bacteriology and Infectious Diseases, Medical School of Kocaeli University, Kocaeli, TURKEY

Summary

IVe aimed to detect whether M. tuberculosis DNA is present in the skin biopsy specimens of various granulomatous skin diseases by polymerase chain reaction. The study group consisted of 4 cases of eiythema induration Bazin (EIB), 2 cases of scrofuloderma, 2 cases of lupus miliaris disseminatus faciei (LMDF), 1 case of metastatic Crohn's disease, I case of Melkersson-Rosenthal syndrome, I case of granuloma annulare and I case of sarcoidosis. The diagnoses were based on the clinical, histopathologic, laboratoiy findings and therapeutic results. M. tuberculosis DNA was present in 3 of the 4 patients with EIB and in I patient with Melkersson-Rosenthal syndrome. M. tuberculosis DNA was not detected in the patients with scrofuloderma, LMDF, metastatic Crohn's disease and granuloma annulare. M. tuberculosis DNA was weakly positive in the patient with sarcoidosis.

The results of this study initiate that M. tuberculosis could have a role in the etiology of EIB. The absence of M. tuberculosis DNA in scrofuloderma could be due to the superinfection or Mycobacteria species other than M.tuberculosis causing scrofuloderma. The detection ofM. tuberculosis DNA in Melkersson-Rosenthal syndrome and absence in metastatic Crohn's disease and granuloma annulare led us to think that furl her studies are required.

Key **Words:** Mycobacterium tuberculosis, Granulomatous skin diseases, Polymerase chain reaction

T Klin J Med Res 1999, 17:59-64

Received: March 17, 1999

Correspondence: Nilgün BİLEN
Yahya Kaptan Konutları
Fil Blok Daire: 18
41050 Kocaeli, TURKEY

Ozet

Bu çalışmada değişik granulomatoz deri hastalıklarında, taze deri biyopsi örneklerinde polimeraz zincir reaksiyonu metodu ile Mycobacterium tuberculosis varlığını araştırmayı amaçladık. Çalışma grubumuzu 4 eritema induration Bazin (EİB), 2 skrofuloderma, 2 lupus miliaris disseminatus fasiei (LMDF), l metastatik Crohn hastalığı, 1 Melkersson Rosenthal sendromu, I granuloma annulare ve 1 sarkoidozis olgusu oluşturdu. Olguların tanıları klinik, histopatolojik ve laboratuar bulgularıyla birlikte tedavi sonuçlarına dayanılarak konuldu.

EİB'li 4 hastanın 3'ünde ve Melkersson-Rosenthal sendromlu I hastada M.tuberculosis DNA'sı saptandı. Skrofuloderma, LMDF, metastatik Crohn hastalığı ve granuloma annulareli hastalarda M. tuberculosis DNA'sı saptanmadı. Sarkoidozisli bir hastada ise M.tuberculosis DNA'sı zayıfpozitif olarak tesbit edildi. Bu çalışmanın sonuçları EİB etiyolojisinde M.tuberculosis'in rolü olabileceğine işaret etmektedir. Skrofuloderma olgularında M.tuberculosis DNA'sının yokluğu siiperinfeksiyona veya M. tuberculosis dışındaki mikobakterilerin etken olmasına bağlı olabilir. Melkersson Rosenthal sendromunda M.tuberculosis DNA'sının saptanması ve metastatik Crohn hastalığı ve granuloma annularede saptananıaması daha ileri araştırmalar yapılması gerektiğini düşündürmüştür.

Anahtar Kelimeler: Mycobacterium tuberculosis, Granulomatoz deri hastalıkları, Polimeraz zincir reaksiyonu

TKlin Araştırma 1999, 17:59-64

Because of the clinical and histopathologic similarities between granulomatous skin diseases (GSD) and skin tuberculosis, Mycobacterium tuberculosis has been implicated to play a role in the etiology of tuberculids and some other GSD.

Detection of M. tuberculosis may indicate a possible etiological role in this group of skin diseases. However, the diagnostic identification of M. tuberculosis in skin diseases has remained difficult using conventional laboratory tests (i.e. microscopy and culture), especially in paucibacillary skin tuberculosis (1,2). Therefore there is a need for rapid and sensitive methods for the detection of M. tuberculosis in skin tissue specimens.

Polymerase chain reaction (PCR) is based on the amplification of DNA or RNA of infectious agents enabling to demonstrate the presence of any particular genetic material, that is low in number or even inactive at the moment. Hence, PCR enhanced the diagnosis of infectious diseases as being a sensitive, specific, rapid and useful diagnostic tool (1). We aimed to detect M. tuberculosis DNA in some of the GSD including cutaneous tuberculosis and erythema induratum Bazin (EIB) by PCR.

Patients and Methods

Twelve (9 female, 3 male, age range 18-79 years) patients with a clinical diagnosis of GSD, who attended to our dermatology department in the period of January 1998 and November 1998 were included in the study. The study group consisted of 4 patients with EIB, 2 patients with scrofuloderma, 2 patients with LMDF, 1 patient with metastatic Crohn's disease, 1 patient with Melkersson-Rosenthal syndrome, 1 patient with granuloma annulare and 1 patient with sarcoidosis.

The diagnoses of EIB and scrofuloderma were based on the presence of relevant personal or familial medical history, clinical appearance, histopathologic examination, strongly positive tuberculin test and well response to the antituberculous chemotherapy. The diagnoses of LMDF was based on the clinical, laboratory findings and histopathologic examination of skin biopsy. The diagnosis of metastatic Crohn's disease was based on the clinical, radiologic, pathologic and endoscopic data. The diagnosis of Melkersson Rosenthal syndrome was based on clinical appearance, histopathologic examination of biopsy specimen obtained from the lip in a patient associated with facial paralysis. The diagnosis of sarcoidosis was based on clinical appearance, histopathologic study of skin biopsy specimen, negative tuberculin test, hilar lymphadenopathy in computerized tomography of the thorax and the high level of serum angiotensin-converting enzyme. The diagnosis of granuloma annulare was based on clinical appearance and histopathologic findings in a patient with diabetes mellitus.

Routine laboratory tests, including complete blood count, standard biochemical analysis, urine analysis and serologic tests for syphilis and HIV infection, tuberculin test, chest x-ray were perfonned in all patients. Bactériologie cultures were perfonned in the cases with scrofulodenna. The culture for M. tuberculosis was unavailable except in one case.

Histopathologic investigation

Punch biopsy specimens were obtained from the representative skin lesions, and the specimens were fixed in "Holland solution" and processed routinely. Acid-fast staining was performed in a total of 8 cases.

Polymerase chain reaction technique

Biopsy specimens were immediately transferred to the laboratory. DNAs were isolated by a modification of the method of Boom et al (3). Briefly, biopsy materials were lysed by incubating at 56°C for one hour in a 500 fxl/ml final concentration of proteinase K and later for half an hour with 4 M Guanidine Thiocyanate, 0.5% N-Lauroylsarcosine. Supernatants were collected after a brief ccntrifugation (2 min at 12.000 g). Nucleic acids were precipitated by 1/10 volume of sodium acetate (3M pH 5.5) and *Vi* volume of 2-propanol at room temperature for 10 minutes. After 10 minutes of centrifugation at 12.000 g, nucleic acid pellets were washed by 70%) cold ethanol, air-dried and resuspended in 50 ml of PCR grade water. Negative samples were always included in the DNA isolation procedures for contamination control. Positive controls were never isolated during these procedures. However, a positive and another negative control were included in eveiy batch of PCR amplifications in order to check master mixture for contamination and activity.

The IS6110 repetitive sequence of "M. tuberculosis complex" genome was the target. PCR tests

were performed in 50 ml volumes. The master mixture was composed of 0.2 uJvl primers each, 1.5 mM $MgCl_2$, 200 mM dNTPs each and 2 units per reaction of Taq DNA polymerase (Boehringer-Mannheim, Mannheim, Germany). To avoid amplicon contaminations, dTTP were replaced by dUTP and 1 U of Uracil-N-glycosylase (UNG) per reaction mixture was supplemented in the master mixtures. Amplification was achieved after 5 min at 50°C and 5 min at 95°C with 40 cycles of 2 min 62°C, 3 min 72°C and 1 min 95°C. Amplified products were never remained at a temperature between 8°C and 60°C to avoid the untoward activity of UNG. A 10 ul portion was run on 2% agarose gel at 100 V and visualized on a UV trans illuminator. However, specific detection of the amplified product was by dot-blot hybridization. The probe (21 base) was 5'-end labeled by digoxigenin by the aid of "5'-End Labeling" kit of Boehringer-Mannheim. Detection of the hybrids were achieved by following the method suggested by the manufacturer of Digoxigenin Detection Kit (Boehringer-Mannheim).

Results

Clinical and laboratory findings

Of 12 patients 3 (2 EIB and 1 scrofuloderma) had a personal or family history of tuberculosis. Tuberculin tests showed an erythematous induration of larger than 1.5X1.5cm. in 4 patients of EIB and 2 patients of scrofuloderma. Tuberculin tests were negative for tuberculosis in the rest of the patients. Serologic tests for syphilis and HIV infection were negative. There was no evidence of pulmonary tuberculosis in 12 patients according to the chest x-ray examinations. Specific culture for M.tuberculosis was available only for one case with scrofuloderma and the result was negative. Bactériologie cultures showed superinfection in two cases with scrofuloderma.

Histopathologic findings

Histopathologic studies revealed a granulomatous inflammation in 7 patients. Ziehl-Neelsen staining for acid-fast bacilli was negative in 8 cases.

Identification of M. Tuberculosis by PCR

M. tuberculosis DNA was present in fresh skin samples obtained from 3 of the 4 patients with EIB and 1 patient with Melkersson-Rosenthal syndrome. We did not determine M. tuberculosis DNA in 2 patients with scrofuloderma, 2 patients with LMDF, 1 patient with metastatic Crohn's -disease and 1 patient with granuloma annulare respectively by PCR. There was weakly positive M. tuberculosis DNA in one patient with sarcoidosis.

Therapeutic results

Three of the 4 cases with EIB and 2 cases with scrofuloderma responded well to fourthly antituberculous therapy. In addition, patients with scrofuloderma were treated with systemic antibiotics because of superinfection findings. We could not follow a patient with EIB (Case 1) and a patient with metastatic Crohn's disease (Case 5). The latter died because of an unknown cause.

The clinical and histopathologic findings, therapeutic results and PCR analyses of the patients are outlined in Table 1.

Discussion

Although PCR is a rather simple concept, sometimes it is complicated in practice by several technical errors. The production of false-positive results by carryover contamination has been observed (1). To prevent this, dUTP and UNG system was incorporated to our PCR mixtures. False negative results may be caused by degraded target DNA, by PCR inhibiting substances present in clinical samples, or by insufficient extraction of DNA (1) due to the difficulty in lysing the lipid-rich cell wall (4). We preferred fresh tissue specimens to avoid the problems caused by the fixation procedures.

The tuberculous origin of EIB remains controversial, especially as attempts to cultivate mycobacteria from the lesions are unsuccessful (5). Although mycobacteria may not be cultured from EIBs, nowadays their DNA may be detected in a majority of cases by PCR (1,5-7). Baselga et al. noted that mycobacterial DNA was detected in 40 of the 52 (77%) biopsy specimens with EIB (5). We

Table 1. Clinical and laboratory findings, therapeutic results, and PCR analyses of the patients with granulomatous skin diseases

Cases	Age and sex	Site	Histologic characteristics	EZN	PCR	Diagnosis	Therapy	Response
1	41,M	lower leg	NSI		+	EIB	AT	not available
2	58.F	lower leg	GI, vasculitis, panniculitis		+	EIB	AT	improved
3	24.F	face	GI			LMDF	isotretinoin	improved
4	58,M	hand	inflammation contains multinuclcar giant cells	-	-	GA	cryotherapy, ILCS	ineffective
5	66,F	abdomen	leucocytoclastic vasculitis		-	Crohn's disease	symptomatic	died
6	47,F	trunk	naked GI		weakly+	sarcoidosis	isotretinoin	no improvement
7	18.F	face	GI			LMDF	doxycyclin	improved
8	48.F	axillae	GI, sinus tract			scrofuloderma	AT	improved
9	50.F	lower leg	GI, vasculitis			EIB	AT	improved
10	20.F	axillae	GI + sinus tract			scrofuloderma	AT	improved
11	64.F	lower leg	panniculitis		+	EIB	AT	improved
12	79,M	upper lip	NSI tissue		+	cheilitis granulomatosa	ILCS	ineffective

EIB, erythema induratum Bazin; AT, antituberculous therapy; GI, granulomatous inflammation; LMDF, lupus miliaris disseminatus faciei; GA, granuloma annulare; ILCS, intralcsional corticosteroid; NSI, nonspecific inflammation.

found M. tuberculosis DNA in 3 of 4 EIB lesions and we think that our results are in agreement with the previous studies. The detection of mycobacterial DNA in EIB suggests the presence of bacilli in the lesions. Whether antituberculous treatment should be administered for EIB is controversial, three of our cases with EIB responded well to the antituberculous treatment. We think that PCR results are consequential, before to start appropriate treatment for EIB in addition to relevant clinicopathologic findings and positive tuberculin test.

Scrofuloderma is a cutaneous form of tuberculosis characterized by subcutaneously located abscess formation and secondary changes of the overlying skin. Ozkan et al. reported DNA amplification products of M. tuberculosis were established by PCR(8). We did not detect M. tuberculosis DNA from 2 patients with scrofuloderma. This result could be due to the superinfection detected in the cases at that time, and we suspected this as a false negative result which could be caused by degradation of M. tuberculosis DNA by bacterial components. However, also other mycobacteria species could be the cause of scrofuloderma.

The pathogenesis of LMDF is still uncertain. Once it was thought to be a variant of lupus vulgaris or a tuberculid, however tuberculous etiology has not been supported by clinical observations, laboratory tests and therapeutic results except histopathology (9,10). Hodak et al could not detect M. tuberculosis DNA in any of the 3 patients with LMDF(4). We also did not detect M. tuberculosis DNA by PCR in our 2 patients of LMDF.

In recent years some reports appeared on the detection of mycobacterial DNA in sarcoidal granulomas by PCR (1,11-16). On the other hand Richter et al. reported 23 samples from patients with sarcoidosis, which all were negative for mycobacterial DNA (17) and Vokurga et al. suggested that M. tuberculosis does not play a pathogenic role in sarcoidosis in most patients (18). We found M. tuberculosis DNA weakly positive in the biopsy specimen from the case with sarcoidosis. Although the pathogenesis of sarcoidosis remains unknown it is possible that some cases of sarcoidosis may result from an immune response initiated by mycobacteria (19), against already nonviable organisms (1). For this reason, we believe that the presence of M. tuberculosis DNA in the skin lesions of sarcoidosis is possible.

It has been shown that M. paratuberculosis DNA exists in intestinal tissue specimens from patients with Crohn's disease (20-22). We did not detect M. tuberculosis DNA in the ulcerative skin le-

sions of our patient with metastatic Crohn's disease. Our PCR technique could not detect M. paratuberculosis.

Since, a variety of causes has been postulated including tuberculosis (23), sarcoidosis and Crohn's disease (24,25) in the etiology of Melkersson-Rosenthal syndrome, we investigated the presence of M. tuberculosis DNA in our patient by PCR. M. tuberculosis DNA was detected in the specimen from the patient with Melkersson Rosenthal syndrome. However, there were no clinical signs and laboratory findings suggestive of tuberculosis, sarcoidosis or Crohn's disease in our case. It is possible that cheilitis granulomatosa is an immune response initiated by M. tuberculosis. In conclusion,we think that screening of M. tuberculosis DNA in Melkersson-Rosenthal syndrome in large series is required.

We could not find a literature about the relationship between granuloma annulare and M.tuberculosis. Here M. tuberculosis DNA was not detected in our patient with granuloma annulare.

Certainly, the detection of M. tuberculosis DNA in various GSD may help to reveal the etiopathogenesis of these dermatoses. PCR is a beneficial method for detecting M. tuberculosis DNA in skin samples of tuberculous origin of GSD. However the results of PCR must be evaluated together with clinical appearance, laboratory findings and sometimes therapeutic response. Our study is a preliminary report and will go on in larger groups of patients.

REFERENCES

1. Dcgitz K. Detection of Mycobacterial DNA in the skin. Etiologic insights and diagnostic perspectives. Arch Dermatol 1996; 132: 71-5.
2. Yates VM, Pormerod L. Cutaneous tuberculosis in Blackburn district (U.K.): a 15-year prospective series, 1981-95. Br J Dermatol 1997; 136: 483-9.
3. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol 1990; 28: 495-503.
4. Hodak E, Trattner A, Feuerman H, Feinmesser M, Tsvieli R, Mitrani R, David M. Lupus miliaris disseminatus faciei-the DNA of Mycobacterium tuberculosis is not detectable in active lesions by polymerase chain reaction. Br J Dermatol 1997;137:614-9.
5. Baselga E, Margall N, Barnadas MA, Coli P, Moragas JM. Detection of Mycobacterium tuberculosis DNA in lobular granulomatous panniculitis (Erythema induratum-Nodular vasculitis). Arch Dermatol 1997; 133: 457-62.
6. Seçkin D, Hızel N, Demirhan B, Tuncay C. The diagnostic value of polymerase chain reaction in erythema induratum ofBazin. Br J Dermatol 1997; 137:1011-2.
7. Chuang YH, Kuo TT, Wang CM, Wang CN, Wong WR, Chan HL. Simultaneous occurrence of papulonecrotic tuberculide and erythema induratum and the identification of Mycobacterium tuberculosis DNA by polymerase chain reaction. Br J Dermatol 1997; 137:276-81.
8. Özkan Ş, Gürler N, Fetil E, Atabey N, Güneş AT. Scrofuloderma. Int J Dermatol 1998; 37: 606-8.
9. Puppin D, Guissas F. Red papules on the face with secondary scarring. Arch Dermatol 1994; 130: 369-74.
10. Dekio S, Jidoi J, Imaoka C. Lupus miliaris disseminatus faciei-report of a case in an elderly woman. Clin Exp Dermatol 1991; 16:295-6.
11. Ghossein RA, Ross DG, Salomon R, Rabson AR. A search for Mycobacterial DNA in sarcoidosis using the polymerase chain reaction. Am J Clin Pathol 1994 ; 101: 733-7.
12. Özkan Ş, Soyal C, Fetil E, Gürler N, Atabey N, İzci Ö, Güneş AT. Sarcoidosis: searching a proof for an old hypothesis. Turk J Dermatopathol 1998;7:48-51.
13. el-Zaatari FA, Naser SA, Markesich DC, Kalter DC, Engstand L, Graham DY. Identification of Mycobacterium avium complex in sarcoidosis. J Clin Microbiol 1996; 34: 2240-5.
14. Popper HH, Kiemen H, Hoefler G, Winter E. Presence of mycobacterial DNA in sarcoidosis. Hum Pathol 1997; 28: 796-800.
15. Fidler HM, Rook GA, Johnson NM, Mc Fadden J, Mycobacterium tuberculosis DNA in tissue affected by sarcoidosis. Br Med J 1993; 306: 546-9.
16. Saboor SA, Johnson NM, Mc Fadden J. Detection of mycobacterial DNA in sarcoidosis and tuberculosis with polymerase chain reaction. Lancet 1992; 339: 1012-5.
17. Richter E, Greinert U, Kirsten D, Rusch Gerdes S, Schlüter C, Duchrow M et al. Assessment of mycobacterial DNA in cells and tissues of mycobacterial and sarcoid lesions. Am J Respir Crit Care Med 1996; 153: 375-80.
18. Vokurga M, Lecossier D, du-Bois RM, Wallaert B, Kambouchner M, Tazi A. Absence of DNA from mycobacteria of the M. tuberculosis complex in sarcoidosis. Am J Respir Crit Care Med 1997; 156: 1000-3.
19. Mitchell DN. Mycobacteria and sarcoidosis. Lancet 1996; . 348:768-769.
20. Lisby G, Andersen J, Engbaek K, Binder V. Mycobacterium paratuberculosis in intestinal tissue from patients with Crohn's disease demonstrated by a nested primer polymerase chain reaction. Scand J Gastroenterol 1994; 29: 923-9.

21. Fidler H M, Thurrel W, Johnson N M, Rook G A, Mc Fadden JJ. DNA associated with granulomatous tissue in Chron's disease. Gut 1995; 36: 942.

22. Clarkston WK, Presti ME, Petersen PF, Zachary PE Jr, Fan WX, Leonardi CL, Vernava AM 3rd, Longo WE, Kreeger JM. Role of Mycobacterium paratuberculosis in Chron's disease a prospective, controlled study using polymerase chain reaction. Dis Colon Rectum 1998; 41: 195-9.

23. Alpert B, Nelson RN. Cheilitis granulomatosa: report of a case. J Oral Surg 1974;32:60-1.

24. Wiesenfeld D, Ferguson MM, Mitchell DN, Mc Donald DG, Scully C, Cochran K, Russell RI. Oro-facial granulomatosis a clinical and pathological analysis. Q J Med 1985; 54: 101-13.

25. Kint A, De Brauwere D, De Weert J, Hendrickx R. Granulomatous cheilitis and Crohn's disease. FJautarzt 1997;28:319-21.