Mycobacterial Strains That Stimulate the Immune System Most Efficiently as Candidates for the Treatment of Bladder Cancer

Article *in* Journal of Molecular Microbiology and Biotechnology · February 2011

DOI: 10.1159/000324331 · Source: PubMed

CITATIONS
20

READS
194

6 authors, including:

Esra Buber
Hacettepe University
40 PUBLICATIONS **57** CITATIONS

SEE PROFILE

Tanil Kocagöz
Acıbadem Mehmet Ali Aydınlar Üniversitesi
117 PUBLICATIONS **1,525** CITATIONS

SEE PROFILE

N. Leyla Acan
Hacettepe University
48 PUBLICATIONS **397** CITATIONS

SEE PROFILE

Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2011;20:24–28
DOI: 10.1159/000324331

Published online: February 19, 2011

Mycobacterial Strains That Stimulate the Immune System Most Efficiently as Candidates for the Treatment of Bladder Cancer

Zehra Stara Yuksel[a] Esra Buber[b] Tanil Kocagoz[d] Alpaslan Alp[c]
Zeynep Saribas[c] N. Leyla Acan[b]

[a]Department of Bioengineering, Faculty of Engineering, Marmara University, Istanbul, Departments of [b]Medical Biochemistry and [c]Medical Microbiology, Faculty of Medicine, Hacettepe University, Ankara, and [d]Department of Medical Microbiology, Faculty of Medicine, Acibadem University, Istanbul, Turkey

Key Words
Anticancer agents · Mycobacteria · Cell wall · Tumor necrosis factor-α · Interleukin-12

Abstract
Background: Intravesical bacillus Calmette-Guérin (BCG) application is widely used in the treatment of superficial bladder carcinoma. Despite being an effective therapy, the pathogenicity and lethal side effects of BCG limits its usage. Intensive research has been carried out to find less toxic and more potent therapeutic agents for the treatment of bladder cancer. Researchers have focused on *Mycobacterium phlei* as an alternative. The cell wall extract of *M. phlei* is sufficient for antitumoral activity. Our preliminary experiments indicate that the fractions rich in cell wall proteins cause activation of tumor necrosis factor (TNF)-α and interleukin (IL)-12. This study aims to identify powerful and less harmful mycobacteria among 88 strains in terms of how they stimulate the immune system. ***Methods:*** Eighty-eight mycobacterial strains were grown in Middlebrook 7H9 medium. The bacterial cells were sonicated after heat treatment. The supernatants were incubated with the monocytic cell line THP-1, followed by measurement of TNF-α and IL-12 response. ***Results and Conclusion:*** In addition to *M. phlei,* the following 12 mycobacterial strains were selected as candidates for superficial bladder tumor treatment: *M. agri, M. aichiense, M. aurum, M. brumae, M. chitae, M. chubuense, M. diernhoferi, M. gadium, M. murale, M. obuense, M. tokaiense* and *M. vaccae*.

Introduction

Bladder cancer is the 4th and 10th most common cancer among men and women, respectively [Jemal et al., 2004]. About 70% of bladder carcinomas are superficial. The factors which develop and progress bladder carcinoma are occupational chemicals; carcinogens; smoking; coffee; pain killers; artificial sweetening agents; parasites; bacterial, viral and fungal infections; bladder stones, and some chemotherapy agents. According to research done by Fidaner et al. [2001], bladder cancer is the 2nd most

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1464–1801/11/0201–0024$38.00/0

Accessible online at:
www.karger.com/mmb

N. Leyla Acan
Department of Medical Biochemistry
Faculty of Medicine, Hacettepe University
TR–06100 Ankara (Turkey)
Tel. +90 312 3051 652/124, E-Mail nla@hacettepe.edu.tr

common cancer type in men over 55 years of age in Turkey. The mortality rate of this type of carcinoma has a lower rank, however, indicating that it is a chronic disease and underlining the importance of the development of novel therapeutics.

Intravesical bacillus Calmette-Guérin (BCG) application is widely used for the treatment of superficial bladder cancer [Morales et al., 1976]. Several studies have shown that the mycobacterial cell wall has an effect both in stimulating the immune system [Filion et al., 2000] and killing the cancer cells [Filion et al., 1999].

There are different treatment alternatives depending on the grade and stage of the tumor. The International Bladder Cancer Group recommends intravesical chemotherapy immediately after surgical operation for low-risk disease. On the other hand, intravesical BCG treatment is the preferred treatment for intermediate- and high-risk disease [Brausi, 2008] and recurrences.

Although BCG is an effective therapeutic, its pathogenicity and lethal side effects such as granulomatous prostatitis, epididymo-orchitis, systemic BCG reactions and allergic reactions limit its use on humans [Brausi, 2008; Lamm et al., 1992]. Moreover, if the patient responds to BCG treatment, prolonged use of it increases the risk of complications.

Intensive research has been carried out to find less toxic and more potent therapeutic agents for the immunotherapy of bladder tumors. Research has focused on *Mycobacterium phlei* as an alternative therapeutic agent. In a trial with 61 patients, it was observed that the cell wall extract of *M. phlei* showed antitumoral activity and had a better toxicity profile as compared to BCG [Morales et al., 2001]. Preliminary experiments with *M. phlei* and *M. smegmatis* showed that the fractions rich in cell wall proteins cause activation of tumor necrosis factor (TNF)-α and interleukin (IL)-12 [Keskin, 2004].

The aim of this study was to determine the most potent and comparatively less harmful mycobacterial species which cause immunostimulation that can be used for anticancer treatment. Cytokine activation by cell wall extracts were measured for this purpose.

Results and Discussion

IL-12 and TNF-α releases by stimulation of monocytic cells with 88 species of mycobacterial cell extracts (table 1) were measured. IL-12 release stimulation activity of most of the extracts was significantly lower as compared to that of BCG ($p < 0.05$). However, stimulation of IL-12 secretion by 9 mycobacterial strains was statistically comparable to that of BCG ($p > 0.05$). On the other hand, stimulation of TNF-α secretion by cell extracts of 23 mycobacterial strains was statistically higher than that of BCG ($p < 0.05$). To select the most promising mycobacterial strains for the treatment of superficial bladder cancer in the future, we eliminated slow-growing and/or pathogenic bacteria. Table 2 shows the significant results obtained for IL-12 and TNF-α release together with the pathogenicity and growth rates of the strains. In light of these findings, we have concluded that in addition to *M. phlei*, 12 of 88 *Mycobacterium* strains that we studied may be alternatives to BCG for immunotherapy of superficial bladder carcinoma. These candidate strains are: *M. agri, M. aichiense, M. aurum, M. brumae, M. chitae, M. chubuense, M. diernhoferi, M. gadium, M. murale, M. obuense, M. tokaiense* and *M. vaccae*.

Regarding antitumoral activity, the best known cytokine is IL-12. It was proven that mycobacterial cell wall extracts increase the secretion of IL-12 from monocytes and macrophages. It was also shown that when IL-12 is used locally or systemically, it has antitumoral activity in many cancer types [Gillies et al., 1998; Izquierdo et al., 1996]. IL-12 shows antitumoral activity by alerting natural killer cells and T lymphocytes, stimulating the secretion of cytokines and inhibiting the angiogenesis [Stern et al., 1996]. TNF-α is responsible for granuloma development, and it was shown that TNF-α inhibited the proliferation of many prostate cancer cell lines [Hillman et al., 1999; Triest et al., 1998].

The highly complex and organized structure of *Mycobacterium* is unique [Brennan, 2003]. When cell walls are disrupted, the free lipids, proteins, lipoarabinomannans and phosphatidylinositol mannosides are solubilized, and the mycolic acid-arabinogalactan-peptidoglycan complex remains as the insoluble residue. These lipids, proteins and lipoglycans may be the signaling effector molecules in the cytokine activation [Chatterjee and Khoo, 1998]. Wang et al. [1995] isolated a boiling water extract from *M. bovis* (BCG vaccine) which had antitumor activity against a murine S180 sarcoma model. The isolate appeared to be a glycan with a molecular mass of 65–87 kDa. The work on isolation of the active fractions in our laboratory of the cell wall extracts of the promising mycobacteria is in progress.

This study presents the immune-stimulating activity of 88 mycobacterial species in an effort to find out a more potent and safer alternative to BCG treatment for superficial bladder tumors. This is a pioneer study, as it is the

Table 1. Mycobacterial strains used in this study

1	*M. abscessus*	ATCC19977	DSM44196
2	*M. agri*	ATCC27406	DSM44515
3	*M. aichiense*	ATCC27280	NT4801
4	*M. alvei*	ATCC51304	DSM44176
5	*M. angelicum*		DSM45057
6	*M. asiaticum*	ATCC25276	NT6101
7	*M. aurum*	ATCC23366	NT4101
8	*M. austroafricanum*	ATCC33464	NT5301
9	*M. bovis (BCG)*	ATCC27289	
10	*M. boenickei*		DSM44677
11	*M. branderi*	ATCC51789	
12	*M. brisbanense*		DSM44680
13	*M. brumae*	ATCC51384	DSM44177
14	*M. canariasense*		DSM44828
15	*M. celatum*	ATCC51130	
16	*M. chimaera*		DSM44623
17	*M. chitae*		NT4301
18	*M. chubuense*	ATCC27278	NT4701
19	*M. conceptionense*		DSM45102
20	*M. concordense*	ATCC BAA-329	DSM44678
21	*M. confluentis*	ATCC49920	DSM44017
22	*M. conspicuum*		DSM44136
23	*M. diernhoferi*	ATCC19340	NT2301
24	*M. doricum*		DSM44339
25	*M. duvalii*		NT4601
26	*M. elephantis*		DSM44368
27	*M. fallax*	ATCC35219	NT5501
28	*M. flavescens*		DSM43219
29	*M. fluoranthenivorans*		DSM44556
30	*M. fortuitum*	ATCC6841	
31	*M. furth*		DSM44567
32	*M. gadium*	ATCC27726	NT5001
33	*M. gallinarum*	ATCC19710	NT5601
34	*M. gastri*	ATCC15754	NT1301
35	*M. gilvum*	ATCC43909	DSM44503
36	*M. goodie*	ATCC700504	DSM44492
37	*M. gordonae*	ATCC14470	NT1101
38	*M. hackensackense*	ATCC BAA-823	DSM44833
39	*M. hassiacum*		DSM44199
40	*M. hiberniae*	ATCC49874	DSM44241
41	*M. holsaticum*		DSM44478
42	*M. houstonense*		DSM44676
43	*M. interjectum*	ATCC51457	DSM44064
44	*M. intermedium*	ATCC51848	DSM44049
45	*M. intracellulare*	ATCC13950	
46	*M. kansasii*	ATCC12478	NT701
47	*M. kubicae*		DSM44627
48	*M. kumamotonense*		DSM45093
49	*M. manitobense*		DSM44615
50	*M. marinum*	ATCC927	DSM44344
51	*M. massiliense*		DSM45103
52	*M. monacense*		DSM44395
53	*M. moriokaense*	ATCC43059	DSM44221
54	*M. mucogenicum*	ATCC	DSM44124
55	*M. murale*		DSM44340
56	*M. neglectum*		DSM44756
57	*M. neoaurum*	ATCC25795	DSM44074
58	*M. nonchromogenicum*	ATCC19530	NT1401
59	*M. obuense*	ATCC27023	DSM44075
60	*M. palustre*		DSM44572
61	*M. parafortuitum*	ATCC19686	NT4006
62	*M. paraseoulense*		DSM45000
63	*M. peregrinum*	ATCC14467,23023	DSM43271
64	*M. petroleophilum*		DSM44182
65	*M. phlei*	ATCC11758	
66	*M. phocaicum*		DSM45104
67	*M. porcinum*	ATCC33775	NT5801
68	*M. pulveris*	ATCC35154	NT7001
69	*M. ratisbonense*		DSM44364
70	*M. rhodesiae*		NT5201
71	*M. salmoniphilum*	ATCC13758	DSM43276
72	*M. scrofulaceum*	ATCC19981	NT1001
73	*M. senegalense*		NT6801
74	*M. senuense*		DSM44999
75	*M. seoulense*		DSM44998
76	*M. septicum*	ATCC700731	DSM44393
77	*M. setense*		DSM45070
78	*M. simiae*	ATCC25275	NT909
79	*M. smegmatis*	ATCC14468	NT2433
80	*M. terrae*	ATCC15755	NT1501
81	*M. tokaiense*		NT4901
82	*M. triplex*		DSM44626
83	*M. trivial*	ATCC23292	NT1601
84	*M. tuberculosis H37RV*	ATCC25618	
85	*M. vaccae*	ATCC29678	
86	*M. wolinsky*	ATCC700010	DSM44493
87	*M. xenopi*	ATCC19250	NT1901
88	*M. yunnanensis*		DSM44838

first work to be tested in its field with a large number of different mycobacterial strains. Although it is important to determine alternative nonpathogenic *Mycobacterium* strains whose cell wall fractions are efficient on bladder carcinoma, this study is only the first step in developing a new anticancer agent. Further study is required, including bladder cell cultures and animal models, to develop suitable agents. In light of these studies, research on other cancer types involving mycobacterial immune stimulation may be initiated.

Table 2. Mycobacterial strains which showed significant TNF-α and/or IL-12 stimulation activity

Strain	TNF-α, pg/μg protein mean ± SD	IL-12, pg/μg protein mean ± SD	Pathogenicity	Growth rate
M. bovis, BCG	3,271 ± 1,549	18.7 ± 6.8	yes	slow
M. agri[a]	7,839 ± 431[b]	30.7 ± 3.5	no	rapid
M. aichiense[a]	4,629 ± 1,902	15.2 ± 0.1[c]	no	rapid
M. aurum[a]	2,058 ± 533	20.7 ± 3.7[c]	no	rapid
M. brisbanense	6,409 ± 3,814[b]	22.8 ± 1.2	yes	rapid
M. brumae[a]	11,068 ± 1,757[b]	7.3 ± 6.3	no	rapid
M. chitae[a]	10,927 ± 812[b]	15.6 ± 10.6	no	rapid
M. chubuense[a]	7,982 ± 786[b]	7.5 ± 3.1	no	rapid
M. diernhoferi[a]	4,199 ± 1,211	19.4 ± 7.1[c]	no	rapid
M. elephantis	11,755 ± 820[b]	12.3 ± 4.6	?	rapid
M. gadium[a]	10,485 ± 1,505[b]	12.2 ± 3.0	no	rapid
M. gallinarum	9,205 ± 1,847[b]	14.9 ± 2.1	?	rapid
M. goodie	10,116 ± 1,229[b]	8.3 ± 3.4	yes	rapid
M. gordonae	7,940 ± 1,316[b]	7.0 ± 3.3	no	slow
M. intracellulare	11,260 ± 1,413[b]	7.2 ± 7.2	yes	slow
M. kansasii	6,900 ± 2,322[b]	8.2 ± 3.8	yes	slow
M. murale[a]	7,259 ± 264[b]	28.4 ± 7.8[c]	?	rapid
M. nonchromogenium	9,476 ± 1,402[b]	14.8 ± 1.9	no	slow
M. obuense[a]	5,054 ± 1,297	14.2 ± 0.4[c]	no	rapid
M. peregrinum	11,684 ± 748[b]	8.7 ± 2.3	yes	rapid
M. phlei[a]	9,908 ± 699[b]	7.6 ± 1.0	no	rapid
M. scrofulaceum	7,894 ± 2,060[b]	18.9 ± 2.4[c]	yes	slow
M. septicum	7,296 ± 819[b]	8.5 ± 5.2	yes	rapid
M. simiae	7,828 ± 379[b]	12.7 ± 2.4	yes	slow
M. terrae	6,895 ± 1,281 [b]	11.4 ± 2.5	no	slow
M. tokaiense[a]	4,025 ± 338	24.4 ± 0.4[c]	no	rapid
M. triplex	10,411 ± 2,137[b]	8.4 ± 2.5	yes	slow
M. tuberculosis H37RV	7,772 ± 1,369[b]	21.7 ± 9.3[c]	yes	slow
M. vaccae[a]	13,617 ± 1,030[b]	11.9 ± 4.2[c]	no	rapid

[a] Selected mycobacteria.

[b] The TNF-α stimulation properties of these mycobacteria were found to be significantly higher as compared to *M. bovis* ($p < 0.05$).

[c] The IL-12 stimulation degree for these mycobacteria was statistically comparable to the stimulation obtained by *M. bovis (BCG)* ($p > 0.05$). The values obtained for the rest of the mycobacteria were statistically lower as compared to *M. bovis (BCG)* ($p < 0.05$).

Experimental Procedures

Material

Löwenstein-Jensen culture media was obtained from Salubris A.Ş. (Turkey), *Mycobacterium* species were obtained from ATCC (American Type Culture Collection; USA) and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen; Germany), THP-1 cells were obtained from the Şap Institute Cell and Virus Bank (HÜKÜK; Ankara, Turkey), penicillin and streptomycin were obtained from Gibco (Gaithersburg, Md., USA), Middlebrook 7H9 broth was obtained from Difco Laboratories (Detroit, Mich., USA), RPMI-1640 medium was obtained from Gibco BRL (Gaithersburg, Md., USA), and IL-12 and TNF-α determination kits were obtained from BioSource (Invitrogen; USA). All the other reagents were analytical grade.

Mycobacterial Strains

Eighty-eight different mycobacterial strains (table 1) were obtained from ATCC, NTCC (National Type Culture Collection; UK) and DSMZ. The species of the strains were checked by PCR-restriction enzyme analysis of *hsp65* using the method described by Telenti et al. [1993].

Mycobacteria were inoculated into Löwenstein-Jensen culture media. They were incubated until colonies were visible. Fresh colonies were then transferred into 50-ml centrifuge tubes containing 10 ml of Middlebrook 7H9 broth culture media. Tubes were incubated in a rotary shaking incubator until the turbidity of each tube reached 1 McFarland. The purity of the cultures was checked with acid-fast staining.

Cell Extract Preparation

Mycobacteria grown in Middlebrook broth were centrifuged at 10,000 *g* for 15 min. The supernatant was discarded. To eliminate residual culture medium, the pellet was washed with 30 ml of 50 mM potassium phosphate buffer, pH 7.4. The final pellet was suspended in 1 ml of phosphate buffer. The suspension was incubated at 95°C for 10 min in a water bath to kill bacteria and to partly disrupt their cell walls [Kent and Kubica, 1985; Winn et al., 2005]. The samples were sonicated for 2 min (at 40% amplitude) with a sonicator (Sonics Vibra Cell VCX 750, USA). Mycobacterial cell wall extracts were stored at –80°C until they were used for further experiments.

Treatment of Monocytic Cell Lines with Mycobacterial Extracts

The monocytic cell line THP-1 was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For the experiments, 500,000 cells/ml/well were dispensed in 24-well microtiter plates in the same medium. Cells were differentiated with 200 nM of phorbol-12-myristate-13-acetate (PMA)/ml for 48 h at 37°C in a humidified atmosphere of 5% CO_2. To examine the effect of mycobacterial extracts, PMA-treated THP-1 cells were treated with mycobacterial extracts (1 μg protein/ml). After 48 h of incubation, the culture supernatants of THP-1 cells were collected, centrifuged at 13,000 *g* and stored at –70°C until determination of TNF-α and IL-12 levels by ELISA. Lipopolysaccharide (1 μg/ml) was used as a positive control [Martha et al., 2001].

Determination of Cytokine Release

The supernatants were collected from the cultures of cells stimulated by lipopolysaccharide or mycobacterial extracts at 48 h and stored at –80°C. Total protein quantity in each sample was determined by the Bradford micro method [Bradford, 1976], with bovine serum albumin as the standard. ELISA for TNF-α and IL-12 (Biosource; Camarillo, Calif., USA) were performed according to the manufacturer's instructions. Triplicate ELISA results were obtained using a 96-well plate reader, Spectra Max M2 microplate reader (Molecular Devices; Canada) [Martha et al., 2001]. Statistics were done using Minitab 13.1 statistical software (Minitab Inc.).

Acknowledgements

This work is part of a project supported by the Ministry of Industry and Trade through the Scientific and Technical Research Council of Turkey (TUBITAK), Project No. SBAG-SANTEZ-5-105S361. It is the MSc thesis of Z.S.Y. Mycobacterial strains were partly provided by Salubris A.Ş., Istanbul. Z.S.Y. received an educational grant from TUBITAK. Z.S.Y., E.B. and N.L.A. received travel grants and E.B., A.A., Z.S. and N.L.A. received honoraria from TUBITAK. T.K. is a consultant for Salubris A.Ş. Z.S.Y. is currently employed by Bios Biomedikal Sistemler San ve Tic Ltd.

The authors express their thanks to Prof. Dr. Orhan Kaya Köksalan in DETAE, Istanbul University Medical Faculty, for *hsp65* PCR-restriction enzyme analysis and confirmation of the species of the strains.

References

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–254.

Brausi M: Approaches to the management of non-muscle invasive bladder cancer: a review of current guidelines and best practice recommendations from the international bladder cancer group. Eur Urol Suppl 2008;7:615–617.

Brennan PJ: Structure, function and biogenesis of the cell wall of *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 2003;83:91–97.

Chatterjee D, Khoo KH: Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. Glycobiology 1998;8:113–120.

Fidaner C, Eser SY, Parkin DM: Incidence in Izmir in 1993–1994: first results from Izmir Cancer Registry. Eur J Cancer 2001;37:83–93.

Filion MC, Filion B, Reader S, et al: Modulation of interleukin-12 synthesis by DNA lacking the CpG motif and present in a mycobacterial cell wall complex. Cancer Immunol Immunother 2000;49:325–334.

Filion MC, Lepicier P, Morales A, et al: *Mycobacterium phlei* cell wall complex directly induces apoptosis in human bladder cancer cells. Br J Cancer 1999;79:229–235.

Gillies SD, Lan Y, Wesolowski JS, et al: Antibody-IL-12 fusion proteins are effective in SCID mouse models of prostate and colon carcinoma metastases. J Immunol 1998;160:6195–6203.

Hillman GG, Triest JA, Cher ML, et al: Prospects of immunotherapy for the treatment of prostate carcinoma – a review. Cancer Detect Prev 1999;23:333–334.

Izquierdo, MA, Degen D, Sypek JP, et al: Antiproliferative effects of interleukin-12 treatment on human tumor colony-forming units taken directly from patients. Anticancer Drugs 1996;7:275–280.

Jemal A, Tiwari RC, Murray T, et al: Cancer statistics, 2004. CA Cancer J Clin 2004;54:8-29.

Kent PT, Kubica GP: Public Health Mycobacteriology, A Guide for the Level III Laboratory. Atlanta, US Department of Health and Human Services, 1985, pp 5–12.

Keskin MS: *M. phlei, M. smegmatis*: the antitumoral effects of nonpathogen mycobacteria species; diss, Hacettepe University, Ankara, 2004.

Lamm DL, van der Meijden PM, Morales A: Incidence and treatment of complications of bacillus Calmette-Guerin intravesical therapy in superficial bladder cancer. J Urol 1992;147:596–600.

Martha M, Oliveira RC, Cristina V: *Mycobacterium bovis* BCG but not *Mycobacterium leprae* induces TNF-a secretion in human monocytic THP-1 cells. Mem Inst Oswaldo Cruz Rio de Janeiro 2001;96:973–978.

Morales A, Chin JL, Ramsey EW: Mycobacterial cell wall extract for treatment of carcinoma in situ of the bladder. J Urol 2001;166:1633–1637.

Morales A, Eidinger D, Bruce AWI: Intracavitary bacillus Calmette-Guérin in the treatment of superficial bladder tumors. J Urol 1976;116:180–183.

Stern AS, Magram J, Presky DH: Interleukin-12 an integral cytokine in the immune response. Life Sci 1996;58:639–654.

Telenti A, Marchesi F, Balz M, et al: Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 1993;31:175–178.

Triest JA, Grignon DJ, Cher ML, et al: Systemic interleukin 2 therapy for human prostate tumors in a nude mouse model. Clin Cancer Res 1998;4:2009–2014.

Wang R, Klegerman ME, Marsden I, et al: An anti-neoplastic glycan isolated from *Mycobacterium bovis* (BCG vaccine). Biochem J 1995;311:867–872.

Winn WJ, Allen S, Janda W, et al: Color Atlas and Textbook of Diagnostic Microbiology, Philadelphia, Lippincott Williams & Wilkins, 2005.