Correlation between Virulence of Various Strains of Mycobacteria and Their Susceptibility to Ethanolic Extract of Propolis (EEP)

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Ethanol extract of propolis (EEP) has antibacterial, antiviral, antiprotozoal and antifungal properties, in addition to many biological effects. Our laboratory has demonstrated a synergistic effect of EEP and antibiotics on the growth of *Staphylococcus aureus*, and suggested that the bactericidal effect of EEP was expressed mainly on virulent mycobacteria rather than on non-virulent (attenuated) ones. The present study was designed to reconfirm the latter finding, by subjecting 17 different mycobacteria strains to EEP, and evaluating whether there is a correlation between the virulence of the mycobacteria strains studied and their susceptibility to EEP. Our findings demonstrate that while the four non-virulent strains studied are not susceptible to EEP, out of the 13 virulent strains studied seven are susceptible and six are resistant to it. These results suggest that while there is no full correlation between virulence of the mycobacteria tested and their susceptibility to EEP, the few strains that were resistant to EEP were non-virulent.

Introduction

Propolis is a natural resinous material produced by honey bees and used by them to strengthen, isolate and disinfect their nests. The ethanolic extract of propolis (EEP) is rich in flavonoid aglycons, phenolic compounds, sesquiterpenes, steroids, amino acids, trace elements and diterpenoids (Wollenweber *et al.,* 1987; Gabrys *et al.,* 1986; Scheller *et al.,* 1989; Matsuno, 1995; Matsuno *et al.,* 1997). EEP has antibacterial, antiprotozoal, antiviral and antifungal activities, as well as ability to accelerate osteogenic processes and to regulate cartilage, dental and bone development (Scheller *et al.,* 1977a&b; Scheller *et al.,* 1968; Starzyk *et al.,* 1977; Scheller *et al.,* 1978, Stojko *et al.,* 1978). EEP has also been shown to have immunostimulatory capacity in animals and patients, to increase the survival rate of mice bearing Ehrlich ascites carcinoma, to exert cytotoxic activity against cancer cells and to protect mice against gamma irradiation (Frankiewicz and Scheller, 1984; Scheller *et al.,* 1988). The latter properties have been related to the antioxidative effect of EEP (Krol *et al.,* 1990). Recently some EEP flavonoids demonstrated ability to inhibit synthesis of protease, integrase and reverse transcriptase of retro viruses (Burke *et al.,* 1995; Critchfield *et al.,* 1996).

Antibacterial activity of EEP gained the highest interest of all biological activities, due to the fact that it is the major use of EEP for the honey bees, and has clinical applicability. Our major findings were (a) demonstrating a synergistic effect of EEP and antibiotics on the growth of the virulent bacteria, *Staphylococcus aureus* (Krol *et al.,* 1993) and (b) suggesting that the bacterial effect of EEP is expressed only on virulent mycobacteria, rather than on non-virulent (attenuated) one (Scheller *et*

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al., 1968). Since the latter finding was performed on a single strain of mycobacteria, and because since that finding a lot of experience has been gained with the antibacterial activity of EEP, we decided to widen the scope of our original study and to evaluate whether there is a possible correlation between the virulence of a variety of mycobacteria strains and their susceptibility to ethanolic extract of propolis *in vitro*.

Materials and Methods

Bacteria and their inoculation

Six standard laboratory strains and eleven wild strains of mycobacteria, isolated from pathological species, were used in this study. The standard strains were: *M.H*$_{37}$*Rv, M.H*$_{37}$*Ra, M.kansasii, M.xenopei*, *M.intracellulare* and *M.BCG*. The wild strains used were: *M*.208, *M*.214, *M*.223, *M*.241, *M*.252, *M*.260, *M*.323, *M*.333, *M*.2563, *M*.2583 and *M*.2976. They were obtained from the Tuberculosis Institute in Warsaw and from the Clinic for Tuberculosis and Pulmonary Diseases of the Silesian Academy of Medicine in Zabrze-Biskupice (Poland), and were stored at 4 °C pending the study. Six-week old mycobacterial cultures were suspended in distilled water, using the Weigls mortar, and were inoculated on Loewenstein-Jensen solid media (containing KH_2PO_4, $MgSO_4$, magnesium citrate, L-asparagine, glycerol, potato flour, egg albumin and malachite green). The concentration of the suspension was established as 1 mg/ml, and the dose inoculated was always $5x10^{-5}$ mg/ml of distilled water.

Preparation of EEP and its incorporation into the medium

Propolis was collected manually in the beehive of the University's farm (bees using mostly poplar buds), and was kept desiccated pending processing. It was extracted in 95% v/v ethanol, in a 1:10 ratio, in hermetically-closed glass vessel, for 10 days at 37 °C, under frequent shaking. The ethanolic extract was then filtered through a Whatman #4 filter paper, and evaporated under vacuum. The dark-brown substance (EEP; yield: 45–60% w/w of raw material) was dissolved prior to use in Loewenstein-Jensen medium. EEP was added to the medium test-tubes, at concentrations of 0, 600, 1,200 and 1,800 μg/ml. The media was then coagulated three times, on three consecutive days, at 75 °C.

Evaluating the virulence of the mycobacteria

At present there is no unequivocally-accepted method to quantify virulence of mycobacteria. To establish a reliable correlation between virulence of mycobacteria and the antibacterial activity of EEP on the same bacteria strains, we used the following seven parameters of bacterial growth and reactivity, and ranked them in order of their virulence-contributing characteristics:

1) Mean growth intensity. The visible growth intensity of each strain was monitored for four consecutive weeks after inoculation in Loewenstein-Jensen media, and was graded at the end of each week as follows: -minus, + (25 colonies), ++ (50 colonies) or +++ (75 colonies and more). Low growth intensity correlates generally with virulence, and rapid growth coincides with non-virulent bacteria (Jacobs and Bloom, 1994).

2) Growth type. Rough (un-smooth, cauliflower-like) growth of mycobacteria was considered as an expression of their virulence, and therefore was ranked "1", while smooth growth was considered non-virulent and was ranked "0".

3) Neutral-red test. This test distinguishes between virulent mycobacteria, which absorb the neutral-red from solution and turn red (ranked "2"), and non-virulent bacteria that do not absorb the red color and remain colorless (ranked "0") (Hein *et al.*, 1958; Kedzia and Koniar, 1980).

4) Susceptibility to isonicotinic acid hydrazide (INH). INH was dissolved in saline and was added to the solid Loewenstein-Jensen media, to yield the following concentrations: 0, 0.025, 0.05, 0.1, 0.2 and 1.0 μg/ml. The majority of INH-resistant mycobacteria were of low virulence and low catalase-peroxidase activity (ranked "0"), while the majority of the INH-susceptible bacteria were virulent, and were ranked "1" (Grange and Davey, 1990; Grange, 1990).

5) Catalase and peroxidase activity – the Bogen test. 0.2% pyrocatechine and 1% H_2O_2 solution is added to the growth medium. Enhanced catalase activity (and consequently formation of bubbles above the mycobacterial colony 30 minutes after its inoculation, indicate virulence (ranked

"1"), while non-virulent strains do not change the color and structure of the colonies, and are ranked "0".

6) Thermolability of catalase activity. Catalase from virulent mycobacteria totally looses its activity upon heating to 60 °C for 10 minutes, while the non-virulent mycobacteria loose its catalase activity upon exposure to temperatures higher than 65 °C (Hein *et al.,* 1958; Kedzia and Koniar, 1980). For comparative purposes, thermolabile catalase of mycobacteria was ranked "1", while the enzyme of thermostable mycobacteria was ranked "0".

7) Susceptibility of mycobacteria to EEP. Lowenstein-Jensen medium containing a fixed concentration of EEP was incubated with mycobacteria, as specified above. After four weeks of incubation, the mycobacteria that were found to be susceptible to EEP (not growing in an EEP concentration of 1,800 μg/ml) were marked "+", and the non-susceptible strains were marked "–".

Results and Discussion

The results of the present study are presented in Table I. In that Table, six standard strains and 11 wild strains isolated from pathological specimen are listed. One of the laboratory strains is considered as the standard strain for virulence ($M.H_{37}Rv$), while $M.H_{37}Ra$ is considered as the standard for non-virulence, also for "attenuation". We referred to all other strains as having "modified virulence".

According to our findings, out of the 17 strains studied, 13 were virulent mycobacteria, and out of them – 7 were susceptible to EEP and 6 were resistant to it. The four attenuated strains were *M.intracellulaire*, M.241, M.252 and $M.H_{37}Ra$, and all of them were resistant to EEP.

In an earlier study from this laboratory we demonstrated a correlation between susceptibility to EEP of the virulent $M.H_{37}Rv$ mycobacterial strain on one hand, and resistance to EEP of the non-virulent $M.H_{37}Ra$ mycobacterial strain on the other (Scheller *et al.,* 1968). Our hypothesis and the distinctive correlation between these two strains and their effects were reaffirmed by others (Grange and Davey, 1990; Suter and Bloch, 1958).

The present study was meant to check whether there was a clear-cut correlation between the two characteristics of the mycobacteria tested, namely – virulence and EEP susceptibility. Our results indicate that within the range of virulence tested, the strains that were susceptible to EEP were equally distributed: 6 susceptible and 7 resistant. The strains of the lowest virulence were resistant to EEP in all concentrations studied.

Table I. The overall virulence of 17 mycobacteria strains, and the correlation of their virulence with their susceptibility to (EEP).

Mycobacteria Strain	Growth Intensity				Growth Type: + = Rough – = Smooth	Neutral-Red Test	Susceptibility to INH	Peroxidase Activity	Catalase Thermo-lability Test	Virulence Ranking	Susceptibility EEP
	1w	2w	3w	4w							
M. 2976	–	+	+++	+++	+	+	+	+	+	6	–
M. 2563	–	–	+	+	+	+	+	+	–	6	+
M. $H_{37}R_V$	–	++	+++	+++	+	+	+	+	–	6	+
M. kansasii	–	+++	+++	+++	+	+	+	+	–	6	–
M. 223	–	+	++	++	–	+	+	+	–	5	+
M. 323	–	+	+++	+++	+	+	–	+	–	5	–
M. 2583	–	+++	+++	+++	+	–	+	+	–	4	–
M. xenopi	–	–	++	++	+	–	+	+	+	4	–
M. BCG	–	–	++	++	+	+	–	–	+	4	–
M. 208	–	+	++	++	+	+	–	–	+	4	+
M. 214	–	+	++	++	+	+	–	–	+	4	+
M. 260	–	+	+++	+++	+	+	–	–	+	4	+
M. 333	–	+	++	++	+	+	–	–	–	4	–
M. intracellulare	–	+++	+++	+++	+	–	–	+	+	3	–
M. 241	–	+	++	++	–	+	–	–	+	3	–
M. 252	–	+	++	++	–	+	–	–	–	3	–
M. $H_{37}R_a$	+	+++	+++	+++	+	–	–	–	–	2	–

The methodological difficulty in assessing virulence of mycobacteria was clear to us when we undertook this study, as all routine methods for evaluating virulence used so far could only yield approximate values. The present study suggests that a combination of evaluation methods for virulence may give a somewhat better quantitation of virulence, but that this terminology is yet not a clear cut one.

The factors inherent to the virulence of mycobacteria are not yet fully defined. It is believed that there might be a genetic code for it (Golanska *et al.,* 1995), and that the mycobacteria might contain an "attenuation indicator lipid", which determines its level of virulence. If we define "virulence" as "the level of pathogenicity of any microorganism enabling it to cause a disease", we will find out that such a level is composed of an endless number of stages, differing in their degree of disease-causing. While "virulence" is considered a variable term, which depends on a variety of factors, only single mycobacteria from virulent-related pairs, such as $H_{37}Rv$-$H_{37}Ra$ or R_1Rv-R_1Ra, where Ra strains are virulent variants of the Rv strains fit this definition. In the present work, some of the attenuated mycobacteria, such as *M.BCG*, which is used for vaccination against tuberculosis, are included in the group of virulent mycobacteria, but are not susceptible to EEP, like the attenuated mycobacteria.

In conclusion, while there is no full correlation between virulence of the mycobacteria tested and their susceptibility to EEP, the few strains that were resistant to EEP were non-virulent, while among the virulent mycobacteria there are strains that are either susceptible or resistant to the EEP.

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