CHARACTERIZATION OF METHANOL EXTRACTION RESIDUE (MER) FROM BACILLUS CALMETTE-GUÉRIN (BCG)

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Analysis of methanol extraction residue (MER) from Bacillus CALMETTE-GUÉRIN (BCG) was carried out to determine some specific chemical compositional characteristics. Samples of MER were found to contain approximately 40% protein and/or peptide, 3% soluble lipids, 17% bound lipids, 8% elemental nitrogen, and less than 2% mycolic acids. Amino acid analysis showed the presence of alanine, glycine and glutamic acid as the major amino acids. The data are reported in terms of the range found for each constituent over the samples analyzed. Somewhat consistent results were obtained between different MER preparations, but notable compositional variations were observed in samples of MER suspensions.

The methanol extraction residue (MER) from Bacillus CALMETTE-GUÉRIN (BCG) has been reported as a non-specific immunostimulant that can suppress the growth of tumor cells introduced into host animals[1]. Owing to potential side effects produced by BCG in patients undergoing immunotherapy[2], and the non-infectious property of MER, the latter has been suggested as a clinical alternative to BCG therapy[2]. HOPPER and PIMM reported that MER, when contacted with tumor cells, may suppress tumor cell growth and extend the number of non-living adjuvants available for immunotherapy[3]. In order to be able to exercise a suitable quality control on MER preparations derived from BCG, data on some of the specific compositional characteristics of MER would be needed. Such data could assist the evaluation of MER suspensions prepared for clinical use. Some related work has been reported on lipid fractionation of the tubercle bacillus[4~7], and several studies have been reported on the chemical analysis of mycobacterial cell wall[8~11]. Specific chemical compositional information of MER however, has not been detailed previously.

We report here some of the compositional characteristics of MER (elemental analysis, soluble and bound lipid content, amino acid composition, total nitrogen and protein, and mycolic acids), with the indication of greater compositional variances in MER suspensions used for clinical treatment. No biological experiments were performed since it was not our intention to correlate compositional changes in a distinct fraction(s) from different batches of MER with variations in biological activity.

Materials and Methods

Methanol extraction residue (MER) samples

Samples of the methanol-insoluble fraction (MER) of phenol-killed acetone-washed Phipps Strain BCG in solid form, and samples of MER suspensions (containing sodium chloride, sodium carboxy-

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methylcellulose, polysorbate and sterile water) were obtained from the Division of Cancer Treatment, NCI, Bethesda, MD, U.S.A.

Chemicals, solvents and standards

Diazomethane was prepared from Diazald (Aldrich Chemicals, Milwaukee, WI). Glass-distilled solvents (Burdick and Jackson, Muskegon, MI) and analytical grade chemicals were used throughout. Standard mycolic acid samples isolated from BCG strain $H_{37}R_V$ were kindly provided by Dr. MEYER GOREN of the National Jewish Hospital, Denver, CO.

Lipids

The methods used for soluble and bound lipids were principally based on those outlined by BRENNAN *et al.*[7]

Soluble lipids

Aliquots (100 mg) of solid MER samples were extracted for 24 hours with chloroform - methanol - water (16: 6: 1) at room temperature. The resultant suspension was filtered, and the insolubles were re-extracted with the same solvent. Both filtrates were pooled, washed with a 2/10 volume of 0.05 N NaCl[12], and concentrated by rotary evaporation to 2~3 ml. The concentrate was then transferred to a dried, tared vial, taken to dryness under nitrogen and vacuum, and weighed. A small amount of benzene was added in the drying step to remove traces of water.

Samples of MER suspensions (equivalent to 100 mg MER) were extracted for 24 hours with chloroform - methanol (16: 6) at room temperature, and the soluble lipid fraction was isolated and determined as described above for solid MER samples. Occasionally it was noted that a slight amount of insoluble matter formed during NaCl washing of the pooled filtrates. In such cases, the NaCl-washed filtrates were evaporated to dryness in a rotary evaporator, and methanol was added to the residue. The methanol-insolubles (representing the soluble lipids) were removed by filtration, dissolved in chloroform, taken to dryness as above, and weighed. It was noted that similar results for soluble lipid content were obtained by either procedure.

Bound lipids

The residue remaining after extraction of soluble lipids was refluxed on a steam bath for 1 hour with 100 ml of chloroform - methanol - water (16: 6: 1) containing 1% HCl, and filtered. The precipitate was re-extracted, and the two filtrates were pooled and washed with a 2/10 volume of 0.05 N NaCl solution[12]. The chloroform layer was concentrated to 2~3 ml, dried under nitrogen and vacuum, and the residue was weighed.

Mycolic acids

Mycolic acids were isolated by refluxing the isolated bound lipids with benzene-methanolic KOH for 24 hours. The reaction mixture was concentrated to a sludge under a nitrogen jet; ether (2 ml/mg bound lipid) was added, and the mixture was refluxed for 2 hours. The ether layer was decanted and the residue re-extracted with ether several times. The ether solutions were pooled, acidified with HCl, and washed with water. The ether layer was then dried over sodium sulfate, evaporated to dryness under nitrogen, and methylated with diazomethane for TLC analysis of the methyl mycolates. Methyl mycolate standards were spotted side-by-side with the sample on a 20×20 cm glass plate coated with a 0.25-mm layer of silica gel G. The plate was developed with hexane - ethyl ether - acetic acid (70: 30: 4), sprayed with concentrated H_2SO_4-H_2O (50: 50), and heated at 100°C for 15 minutes. Orange spots were observed under long wavelength UV light. The Rf values of standard methylmycolates I and II were found to be 0.91 and 0.95, respectively.

Elemental nitrogen

Nitrogen content of MER samples were determined with a Perkin-Elmer 240 Elemental Analyzer connected to a Perkin-Elmer Micro Analytical Computer MC-1. The combustion temperature was 950°C and the reduction temperature was 500°C. Samples of MER suspensions were analyzed after drying at 110°C to a constant weight. Percent nitrogen was calculated relative to weight of solid for solid MER samples, or to dried solids weight for samples of MER suspensions.

Amino acid analysis

Five mg of solid MER (or its equivalent in samples of MER suspensions) were hydrolyzed with 1 ml methane-sulfonic acid in a sealed tube in nitrogen at 100°C for 24 hours. The solution was filtered, 1 ml of 3.15 N NaOH was added, and the volume of the filtrate was adjusted to a concentration of 1 mg/ml with water. A 40-μl aliquot of this solution was taken for analysis of total amino acids on a Durrum D-500 amino acid analyzer.

Solid content of MER suspensions

The solids content in samples of MER suspensions was estimated by evaporation of sample aliquots in a forced-air oven to constant dry weight. Solids content of the suspending medium used to prepare MER suspensions was estimated in the same manner.

Results

Characterization of MER

Amino acid analysis of MER showed the presence of 14 amino acids, with alanine, glycine and glutamic acid as major constituents (Table 1). The precision in amino acid analysis determined from eight replicate analyses of a single MER sample showed an average percent standard deviation over all amino acids measured in the order of 7%. The amino acid profile was found to be fairly constant for the three MER samples obtained for this study. The standard deviation for these three MER samples ranged from 0.01 (glutamic acid and phenylalanine) to 0.12 (glycine) μmoles/mg MER. Total MER protein, based on the sum of the wt-% of the 14 amino acids, ranged from 35~46%.

Table 1 also indicates the range of MER levels determined for soluble and bound lipids, mycolic acids, and total nitrogen. The average wt-%s (± standard deviation) of these constituents were 3.1 (±0.2), 16 (±1.5), 1.6 (±0.01), and 7.6 (±0.8), respectively. The total (soluble plus bound)

Table 1. Chemical composition of MER of BCG.

Constituent	Concentration range found*
Soluble lipids	2.9~3.3
Bound lipids	15~18
Mycolic acids	1.5~1.6
Total nitrogen	7.4~7.9
Total protein**	35~46
Amino acids	**μmoles/mg MER**
Alanine	0.46~0.64
Glycine	0.29~0.53
Glutamic acid	0.40~0.42
Aspartic acid	0.30~0.41
Leucine	0.25~0.38
Valine	0.24~0.36
Proline	0.16~0.24
Methionine	0.11~0.21
Arginine	0.12~0.19
Lysine	0.11~0.19
Isoleucine	0.11~0.14
Histidine	0.04~0.09
Serine	0.01~0.08
Phenylalanine	0.02~0.05

* Wt.-% in MER.
** From amino acid analysis.

Table 2. Chemical analysis of MER in suspensions.

Constituent	Concentration range found*
Soluble lipids	5~15
Bound lipids	14~24
Mycolic acids	1.2~1.6
Total nitrogen	7.2~11
Total protein**	1.2~19
Amino acids	**μmoles/mg MER**
Alanine	0.06~0.40
Glycine	0.01~0.31
Glutamic acid	0.01~0.31
Aspartic acid	0.01~0.25
Leucine	0.01~0.21
Serine	0.01~0.14
Methionine	0.01~0.07
Histidine	<0.01~0.07

* Wt.-% in MER.
** From amino acid analysis.

lipid content of MER was estimated to be 20~24%, while mycolic acids averaged roughly 10% of the bound lipids.

Analysis of MER in Suspensions

Only eight amino acids were detected in samples of MER suspensions (Table 2). Two noteworthy observations were that (a) the total MER protein estimated from the amino acid results was found to be significantly lower than that determined for MER solid samples, and (b) the variation between samples of MER suspensions was significantly greater than that noted between samples of solid MER (average coefficient of variation for amino acid analysis of MER in suspensions was 97%, *versus* 19% for solid MER). Alanine, glycine and glutamic acid were also found to be major amino acids in samples of MER in suspensions.

The higher concentrations found for soluble lipids for MER in suspensions may be due to some co-extraction of suspension additives. With the exception of soluble lipids and protein (from amino acid analysis), other constituents measured (bound lipids, mycolic acids, total nitrogen) for MER were similar for both solid MER and MER in suspensions. Nonetheless, the data variances were substantially greater for measurements of constituents of MER suspension samples (Tables 1 and 2). The solids content of MER suspension samples ranged from 21~28 mg/ml (average: 24±3 mg/ml).

The vehicle used in the preparation of MER suspensions was separately analyzed. The results of an analysis of a 20-ml aliquot of the vehicle indicated the absence of amino acids and nitrogen-containing components, however, a small amount of material was extracted in lipid fraction. The solids content of the vehicle sample was determined to be 15 mg/ml. The MER was estimated to be in the order of 40% of the total solids in the MER suspension samples.

Discussion

Several studies have been reported on the chemical analysis of mycobacterial cell wall[8~11], but none thusfar on the chemical characterization of MER derived from BCG. As a consequence, no analytical tests have been available for the evaluation of reproducibility of MER samples derived from BCG preparations. We report here some of the compositional characteristics of MER derived from BCG which might be useful in evaluation of MER batch reproducibility. Our results also indicate that problems may exist in preparing uniform MER suspensions.

The major amino acids of mycobacterial cell wall skeleton (CWS-1) have been reported to be glutamic acid and alanine[8], which are also two of the major amino acids (along with glycine, aspartic acid, leucine and valine) which were found in the MER of BCG (Table 1). Aspartic acid, glycine and leucine (in addition to serine and threonine) were minor amino acids in CWS-1[8]. Our results also indicate that more amino acids, as well as higher concentrations of specific amino acids, are detectable in solid MER than in samples of MER suspensions. The mycolic acids content in MER (10~11%) was determined from the bound lipid fraction. This value was much smaller than that found (97%) from the total lipids of CWS-1[8].

Our data indicate a much greater consistency for analyses of MER solid samples than for analyses of samples of MER in suspensions. Immunotherapeutic studies of mice treated with MER found that depression of tumor growth and prolongation of host survival occurred in only a few instances[13]. Whether observances like this are due to biological variation or to relative variances in the MER employed is not known, yet the standardization of MER suspensions to greater uniformity might effectively reduce the potential for bias in the biological results from this vector. Furthermore, it is well known that it is essential to maintain strict environmental control of a fermentation[14]. The changing of C/N ratios and mg content can markedly effect the chemical composition of microbes. Very subtle media and environmental changes can cause notable changes in amino acid content for a cell. Thus,

strict fermentation parameters must be followed in the production of BCG to insure highly reproducible material.

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