HUMAN AND RAT LUNG BIOTRANSFORMATION OF CYCLOSPORIN A AND ITS DERIVATIVES USING SLICES AND BRONCHIAL EPITHELIAL CELLS

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ABSTRACT:
Lung biotransformation of the immunosuppressants, cyclosporin A (CSA), the hydroxyethyl derivative SDZ IMM 125 (IMM), and the methylcarbonate derivative SDZ SCP 764 (SCP), was demonstrated in slices from human and rat. The major biotransformation pathway for CSA and IMM (0.1–10 μM) was hydroxylation at amino acid 1 to form AM1 or IMM1, while for SCP it was an esterase cleavage of the methylcarbonate group to form AM1 in both species. The initial rate (0–1 hr) of human total metabolite formation increased proportionally with substrate concentration. AM1 formation was five times greater from SCP, an esterase pathway, than CSA, an oxidative pathway which was inhibited (50%) by ketoconazole. At 24 hr human lung CSA metabolite formation was greater than IMM (3-fold) or SCP (2-fold), whereas rat lung and liver and human bronchial epithelial cell SCP metabolite formation generally exceeded CSA or IMM metabolism.

CSA biotransformation is expected to occur throughout the human lung as demonstrated by the similar metabolite profile and extent of metabolism by slices derived from five different regions. The scaling of slice total metabolism to organ metabolism revealed that initially lung CSA metabolite formation would be equal to liver but with time liver metabolism would exceed lung for human and rat.

This study has demonstrated that human and rat lung are metabolically active, exhibiting oxidative and esterase pathways toward cyclosporin derivatives. The lung will play an important role in this metabolism, particularly when administered via inhalation; however, the liver will also be a major organ involved in the total clearance of these compounds.

Bronchial asthma, hypersensitivity pneumonitis, and lung allograft rejection are generally characterized by an infiltration of inflammatory cells. The involvement of T lymphocytes in the late asthmatic response is suggested by the increased number of activated CD4⁺ T cells in broncho-alveolar lavage fluid and bronchial biopsy specimens from asthmatic subjects (1). Therapeutic intervention has included the regulation of T-cell derived cytokine production, which in turn affects eosinophil and mast cell survival and infiltration. Lung allograft recipients often develop progressive bronchiolitis obliterans, for which the cause is unknown and which is treated with the same immunosuppressive agents as acute rejection (2).

The immunosuppressant cyclosporin A (CSA)¹ is effective through its action of inhibiting T-cell activation and cytokine production in animal models of hypersensitivity (3–5). Human mononuclear cells derived from patients with bronchial asthma were less responsive in the presence of CSA to interleukin-2 or phytohemagglutinin stimulation (6, 7). Additionally, significant decreases in the production of granulocyte/macrophage colony-stimulating factor and interleukin-5 resulted in a decreased eosinophil activity and proliferation (7). The clinical benefit of CSA therapy has been shown in patients with severe chronic bronchial asthma by an improvement of pulmonary function, as monitored by the peak expiratory flow rate, and it has been associated with a decrease in serum interleukin-2 receptor levels (8, 9).

Administration of CSA directly to the lung as an aerosol would increase the local immunosuppression within the lung and decrease CSA systemic exposure, as well as reduce the risk of developing renal and liver side effects. Data to support the improved efficacy of aerosolized CSA and safety was shown for the prevention of lung allograft rejection in rats and dogs (10, 11). Delivery of aerosolized CSA in humans has been shown with single and double-lung allograft recipients exhibiting acute persistent graft rejection and bronchiolitis obliterans (12).

Two additional cyclosporins that exhibit a similar potency to CSA and a potentially wider safety margin include the hydroxyethyl derivative, SDZ IMM 125 (IMM), and the methylcarbonate derivative, SDZ SCP 764 (SCP) (fig. 1). IMM differs from CSA in that it is less metabolically active, exhibiting oxidative and esterase pathways toward cyclosporin derivatives. The lung will play an important role in this metabolism, particularly when administered via inhalation; however, the liver will also be a major organ involved in the total clearance of these compounds.

¹ Abbreviations used are: CSA, cyclosporin A; IMM, SDZ IMM 125; SCP, SDZ SCP 764; AEBSF, [4-(2-Aminoethyl)benzenesulfonyl]fluoride, HCl; EBSS, Earle’s Balanced Salt Solution MEM, Minimum Essential Medium; BME, Basal Medium Eagle; CYP, cytochrome P450; CYP3A, cytochrome P4503A; HBE, human bronchial epithelial cells.

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The site of primary metabolite formation and amino acid number within the cyclic undecapeptide structure is denoted. The location of the tritium radio nuclide was the same for each compound.

Materials and Methods

Chemicals. [3H]CSA [Mebmt-β-3H]cyclosporin A with a specific activity of 10.7 Ci/mmol was obtained from Amersham (Buckinghamshire, UK). [3H]SDZ IMM 125, [2-hydroxy-ethyl-D-250 mmol, [3H]SCP 764, [8-methylcarboxyl][Mebmt-β-3H]cyclosporin with a specific activity of 3.8 Ci/mmol, and unlabeled CSA, IMM, and SCP were prepared at Sandzopharma Ltd. (Basel, Switzerland). The radiochemical purity of each compound was checked by HPLC and was greater than 98% for CSA and IMM, and 96% for SCP. Tissue culture media components including Glutamax were purchased from Gibco (Grand Island, NY) and Basel, Switzerland, and the Mito+ serum extender and NuSerum from Collaborative Biomedical Products/Becton Dickinson (Heidelberg, Germany). The protease inhibitors aprotinin and AEBSF were purchased from Boehringer Mannheim (Rotkreuz, Switzerland) and Calbiochem (Lucerne, Switzerland), respectively. A low melting agarose (25–30°C) was obtained from Serva (Heidelberg, Germany). The Cell Death Detection Elisa Kit was obtained from Boehringer Mannheim (Mannheim, Germany), and ketoconazole was obtained from Janssen Biotech (Olen, Belgium). All other reagents were of the highest grade available and were purchased from commercial sources.

Human Lung Slice Preparations. Human lungs (N = 6), obtained through the Association of Human Tissue Users (Tucson, AZ), were cored transversely (fig. 2). The slices (12.1 ± 2.5 mm thick in thickness, 13.2 ± 0.9 mg wet weight, 1.8 ± 1.2 mg slice protein. The rat slices were maintained at 37°C in a humidified incubator with circulating 5% CO2-air mixture in roller cultures (1 slice/insert) in 2 ml of the EBSS slicing buffer (pH 7.4) additionally fortified with 2 mM Glutamax, 0.2% Mito+ serum extender, 1 mM dexamethasone, 25 mM nicotinamide, 10 mM glucagon, 0.1 mM Δ-aminolevulinic acid, 0.3 μM aprotinin, 25% antibiotic/antimycotic solution, 10% NuSerum, and glucose (25 mM) in place of fructose and mannitol.

Human Bronchial Epithelial Cells. Normal human bronchial/ tracheal epithelial cells were obtained from Clonetics (San Diego, CA). The cells were cultured in 24-well Falcon culture plates in bronchial epithelial growth medium (BEGM, Clonetics) supplemented with retinoic acid and bovine pituitary extract and maintained at 37°C in a humidified 5% CO2-air mixture.

Biotransformation. Lung biotransformation was evaluated by HPLC from slice incubations of 0.1, 1, or 10 μM [3H]CSA (1 μCi/ml for 0.1 μM, or 3.2 μCi/ml, [3H]IMM (0.8 μCi/ml for 0.1 μM or 3.8 μCi/ml), or [3H]SCP (0.38 μCi/ml for 0.1 μM or 3.8 μCi/ml). The biotransformation of [3H]CSA (1 μM) was also evaluated in the presence of ketoconazole (2 μM) in human lung slices, which was added 30 min prior to CSA. All compounds were dissolved in dimethylsulfoxide which had a final culture concentration of 0.1%, or 0.2% for the ketoconazole studies. The stability of CSA, IMM, and SCP was assessed in the absence of a slice for the duration of the culture period.

Sample collection and extraction from slices were made following the method described by Vickers et al. (24). For the human bronchial epithelial cells, the medium was collected and the cells scraped in the presence of methanol (0.5 ml).

All fractions were pooled to a final volume of 1 ml containing 30% methanol. Prior to HPLC analysis the sample proteins were pelleted by centrifugation (60,000g) for 10 min at 25°C, and the supernatant collected for injection (200 μl). Slices protein was determined by the method of Bradford (25) using bovine immunoglobulin as the standard.

HPLC analysis of samples derived from [3H]CSA and [3H]IMM incubations followed the conditions previously published (24, 26).

The samples derived from [3H]SCP incubations were analyzed following the CSA HPLC conditions (24). Sample radioactivity was
monitored on-line with a Berthold LB 507A radioactivity monitor (Berthold, Wildbad, Germany). The cyclosporin derivatives and metabolites were identified according to the known retention times of parent and reference metabolites and by comparison with biological reference samples. Quantification of the cyclosporin derivative and metabolites was achieved by integration of the peak areas using the Hewlett Packard or Kontron data system.

The stability of CSA was 98% after 24 hr and 93% after 48 hr of incubation, while the stability of IMM was 99% after 24 and 98% after 48 hr of incubation in the absence of a slice. The stability of SCP was 93% after 24 hr, with 4% of the radioactivity co-eluting with AM1, and 88% after 48 hr with 9% co-eluting with the AM1 peak, which was subtracted from AM1 formation.

Viability. The slice viability of control and CSA, IMM, and SCP (0.1, 1, and 10 μM) exposed human lung slices was determined by slice protein synthesis and ATP content as described by Fisher et al. (27).

Human bronchial epithelial cell, rat liver, and lung slice viability was determined by the leakage of histone-associated DNA fragments, mono- and oligonucleosomes using the Cell Death Detection ELISA Kit from Boehringer. Aliquots (100 μl) of slice and cell homogenate prepared in 500 μl EBSS and diluted to 100 ng protein/100 μl with incubation buffer were added to the microtiter plates. The medium (100 μl) was analyzed straight. The per cent leakage of DNA fragments was calculated from the optical density of medium over the total (slice or cell homogenate plus medium).

Results

Metabolite Patterns. The biotransformation of [3H]CSA, [3H]IMM, and [3H]SCP at 0.1, 1, and 10 μM was investigated in human lung slices, human bronchial epithelial cells, and rat lung and liver slices. The major metabolite formed by human lung slices from all three compounds was a hydroxylated metabolite at amino acid 1 (fig. 3). This metabolite has been previously characterized in human liver slices as AM1 for CSA and IMM1 for IMM. (24, 26). The other known CSA primary liver metabolites, hydroxylation at amino acid 9 to form AM9, and the N-demethylated metabolite at amino acid 4, AM4N, were also formed but in very low amounts. For IMM, the analogous primary metabolites were more extensively formed.

The human bronchial epithelial cells metabolized each compound; however, low amounts of the primary metabolites of CSA and IMM were evident, indicating that these metabolites were rapidly converted to further secondary metabolites. For SCP the major metabolite formed was AM1 (data not shown).

The metabolite profile of CSA and IMM from rat lung slices revealed low amounts of the primary hydroxylated and N-demethylated metabolites, the highest metabolite formed being AM1 and IMM1, respectively. Liver slices produced all the primary metabolites as described previously (24, 26). For SCP the major metabolite formed by rat lung and liver slices was AM1 (fig. 4).

The metabolism of CSA by both human and rat lung resulted in the formation of several polar peaks. These peaks have not been identified, and there is a difference in the production depending on the tissue studied. In the lung, the appearance of these polar metabolites occurs within the first hour of culture, while in the liver the polar peaks appear after several hours of culture time.

Biotransformation. The initial rate (0–1 hr) of CSA total metabolite and AM1 formation by human lung slices increased proportionally from 0.1 to 10 μM substrate concentrations. The rate of AM1 formation derived from SCP was in general five times greater than that derived from CSA, and AM1 formation represented 13–85% of the SCP metabolites compared with 7% or less from CSA. The rate of IMM metabolite formation was generally lower than CSA (table 2). Lung slice metabolite formation was linear from 1–48 hr with total metabolite formation paralleling AM1 formation in the CSA cultures. The rapid rate of initial total metabolite formation from 0–1 hr was evident in both the human and rat lung slices (fig. 5) and not in rat liver slices. Metabolite formation of IMM and SCP was also linear with the time (data not shown).

The biotransformation of CSA by the human lung slices was CYP dependent, as shown by slices derived from region 1 of two human lungs and incubated in the presence of [3H]CSA (1 μM) and ketoconazole (2 μM). Formation of AM1 was inhibited ~50% in the presence of ketoconazole at 2 and 4 hr post-[3H]CSA addition compared with slices exposed to [3H]CSA only. By 24 hr of culture, AM1
formation in the presence of ketoconazole was still decreased as much as 25% compared with CSA alone (data not shown).

The mean extent of CSA total metabolite formation and AM1 formation by the human lung slices, the HBEC, the rat lung, and liver slices increased proportionally when the substrate concentrations were increased from 0.1 to 10 μM (table 3). In accordance with a greater rate of CSA metabolite formation by the human lung was a larger amount of CSA total metabolite formation at 24 and 48 hr compared with IMM and SCP. IMM metabolite formation represented about 25% of CSA at 10 μM substrate concentrations. The extent of SCP metabolite formation was lower than CSA (∼50%) and higher than IMM metabolite formation (fig. 6).

The importance of the involvement of an esterase reaction in the formation of AM1 from SCP versus an oxidative pathway from CSA was apparent in the human bronchial epithelial cells. SCP total metabolite formation generally exceeded CSA metabolite formation twofold. AM1 formation from SCP was about 13-fold greater at 0.1 μM and 17-fold greater at 10 μM substrate concentrations compared with CSA AM1 formation. These results could indicate that the esterases were more prominent than the oxidative enzymes in the human bronchial-derived cells as compared with the human lung slices (table 3).

In the rat, the mean initial rate (1 hr) of CSA (1 μM) total metabolite formation by lung slices was 452 ± 134 pmol/hr/mg slice protein, which was 14 times greater than CSA total metabolite formation by liver slices, 32 ± 4 pmol/hr/mg slice protein. The extent of CSA total metabolite formation increased proportionally from 0.1 to 10 μM, and was about twofold greater in the lung than in the liver; however, the extent of AM1 formation was greater in the liver, 7-fold at 0.1 μM and 1.3-fold at 1 and 10 μM. IMM metabolite formation by the rat lung and liver slices was generally comparable and lower than CSA. Biotransformation of SCP by rat liver slices exceeded lung slice metabolism and CSA liver metabolism. The main metabolite of SCP, AM1, represented up to 63% of the total metabolites in lung and up to 73% in liver (table 3).

Lung Regions. The regional biotransformation of [3H]CSA (10 μM, 2, 24, and 48 hr) was investigated in slices derived from different regions of two human lungs, as shown in fig. 2. The metabolite profile was the same in all regions and similar to the radiochromatogram shown for region 1 in fig. 3, demonstrating that the primary hydroxylated and N-demethylated metabolites and several secondary metabolites would be formed throughout the lung. The extent of total metabolite formation was also similar for the various regions. The viability of the slices from the different lung regions, as assessed by protein synthesis and ATP levels at 24 and 48 hr, were also comparable (data not shown).

Upscaling. The relative contribution of the lung first-pass effect of [3H]CSA as compared with that of the liver was estimated by scaling the initial rate of total metabolite formation (μmol/mg slice protein) in the slices (mg wet weight) to the whole organ, based on the weight of the organ (μmol total metabolite/organ). Lung tissue represents about 0.7% of the body weight in man and 0.6% in the rat, while liver represents 2–5% of the body weight in man and 3.5% in the rat (28–30). Scaling of the 24-hr total metabolite formation data was done to determine the relative contribution of the lung to the total biotransformation of [3H]CSA (systemic and presystemic metabolism) as compared with that of the liver.

For both man and rat, the initial lung biotransformation of [3H]CSA just after administration (first-pass effect) is predicted to exceed that...
of liver, whereas total liver biotransformation of \([3\text{H}]\text{CSA}\) is predicted to exceed that of the lung. In man, lung CSA initial metabolite formation was predominant, 1.5-fold greater at 1 \(\mu\text{M}\), as compared with that of liver, but liver total metabolite formation was 8-fold greater at 1 \(\mu\text{M}\), as compared with the lung. In the rat, the initial metabolism formation of \([3\text{H}]\text{CSA}\) was 1.8-fold higher in the lung compared with the liver, but the total extent of liver CSA metabolite formation was about 4-fold greater at 1 \(\mu\text{M}\) \([3\text{H}]\text{CSA}\) than the lung biotransformation of CSA (table 4).

Viability Parameters. Protein synthesis in the human lung slices was linear over the 48-hr culture, virtually doubling between 24 and 48 hr (fig. 7). No significant effects on protein synthesis were caused by CSA, IMM or SCP exposure. ATP levels in lung slices derived from different humans ranged from 12 to 38 nmoles ATP/mg slice protein and remained stable throughout the 48-hr culture period. There were no significant differences in the lung slice ATP content by CSA, IMM, or SCP treatment (data not shown).

The viability of the human bronchial epithelial cells and rat lung and liver slices was characterized by the leakage of histone-associated DNA fragments, mono- and oligo-nucleosomes into the medium. There were no significant increases resulting from CSA, IMM, or SCP treatment (data not shown).

Discussion

This study demonstrates that both human and rat lungs would metabolize cyclosporin A, its hydroxyethyl derivative IMM, and the ester-derivative SCP. Precision-cut lung slices were used to facilitate the comparison of compound biotransformation in human and rat lungs and rat liver.

The biotransformation pathway of the cyclosporins CSA and IMM in the lung was similar to that of liver in that the primary hydroxylated and N-demethylated metabolites previously reported for liver slices were also produced by lung slices of both human and rat (24, 26). Differences, however, in the proportion of the primary metabolites formed by the lung slices as compared with the liver slices were evident. The hydroxylated metabolite at amino acid 1 (AM1 for CSA and IMM1 for IMM) was clearly the major primary metabolite formed by human lung, while only small amounts of the hydroxylated metabolite at amino acid 9 (AM9 for CSA) and the N-demethylated metabolite (AM4N for CSA and IMM4N for IMM) were formed. Both human and rat lung slices revealed a rapid formation of polar peaks from CSA which are usually evident in liver slices at later incubation times (25). The structures of these polar peaks have not been elucidated. We have also found these metabolites in lung and liver microsomal incubations from human and rat, but not in incubations from dead slices (data not reported) or stability samples, indicating that the polar peaks represent metabolites.

The initial rapid rate of total metabolite formation in human and rat lung slices as compared with that in liver is partly a result of the more rapid formation of the polar peaks. Additionally, the more permeable nature of lung tissue could lead to a more rapid accessibility of the compound to the xenobiotic metabolizing enzymes, yielding a more rapid initial rate of metabolism. Metabolite formation was linear with the culture time, indicating that the metabolic functionality and viability of the slices was good.

CSA metabolite formation in lung slices was generally greater for

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TABLE 2

<table>
<thead>
<tr>
<th>Rate of total metabolite (TM) and AM1 formation</th>
<th>Initial Rate of TM Formation (pmol/mg protein/hr)</th>
<th>Initial Rate of AM1 Formation (pmol/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSA</td>
<td>IMM</td>
</tr>
<tr>
<td><strong>Human lung 1</strong></td>
<td></td>
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</tr>
<tr>
<td>0.1 (\mu\text{M})</td>
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<td>1 (\mu\text{M})</td>
<td>90.43</td>
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<tr>
<td>10 (\mu\text{M})</td>
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<tr>
<td><strong>Human lung 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 (\mu\text{M})</td>
<td>10.30</td>
<td>10.71</td>
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<tr>
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</tr>
<tr>
<td>10 (\mu\text{M})</td>
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<tr>
<td><strong>Human lung 3</strong></td>
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<tr>
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<td>1 (\mu\text{M})</td>
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<tr>
<td>1 (\mu\text{M})</td>
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</tr>
<tr>
<td>10 (\mu\text{M})</td>
<td>1273.48</td>
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</tr>
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ND: not determined.
Esterase cleavage followed by a rapid degradation of the ester group to form the nonimmunosuppressive metabolite AM1. The partial inhibition of AM1 formation from CSA by the CYP inhibitors ketoconazole indicates the involvement of CYP enzymes in human lung biotransformation of CSA.

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The partial inhibition of AM1 formation from CSA by the CYP enzymes involved.

The major biotransformation pathway of SCP was cleavage of the ester group to form the nonimmunosuppressive metabolite AM1. The design of SCP predicted a local immunosuppressive effect in the lung, followed by a rapid degradation via esterases, so that the systemic exposure to the parent compound would be low. Esterase cleavage

CSA than IMM as reported for human and rat liver (26). Formation of the primary hydroxylated and N-demethylated metabolites of CSA and IMM are known to be CYP3A dependent in human liver (31, 32). The partial inhibition of AM1 formation from CSA by the CYP inhibitors ketoconazole indicates the involvement of CYP enzymes in human lung biotransformation of CSA.

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from the lung into the systemic circulation with the slice cultures, nor is it possible to predict the proportion of compound reextracted from the systemic circulation by the lung. Highly lipophilic compounds such as CSA may exhibit a long lung residency, in which case lung metabolism could contribute substantially to the clearance of the compound from the respiratory tract (17). Additionally, the lung receives 100% of the cardiac output as compared with 25% for the liver, increasing the possibility for lung reextraction of such lipophilic compounds. The rat oral and iv in vivo studies, however, revealed that liver extraction of both CSA and IMM was greater than lung extraction. Lung CSA levels were about 20-fold lower than liver levels after a single oral or iv dose, and 31-fold lower following multiple oral doses, 10 mg/kg/day for 21 days (34). For the derivative IMM, lung levels were also lower, about 4-fold, as compared with liver levels after an iv dose for 10 days (14).

The isolated human epithelial cells used in this study were derived from the conducting bronchial/tracheal airways and exhibited biotransformation capability toward the cyclosporin derivatives even though esterase reactions were more prominent than oxidative in comparison those of the lung slices. In the lung the major cytochrome P450s identified in man include 1A1, 2A6, 2C9, 2E1, and 3A4, and in rat they include CYP1A, CYP2B, and CYP3A (19, 21). The respiratory tract is a more heterogenous system than the liver, composed of a wide variety cell types, and the biotransformation enzymes are distributed nonuniformly within each segment. Methods for the isolation and culture of the various cell types are not developed across different species, including human. Hence, the methodology for preparing organ slices is ideal for investigating lung biotransformation and function in various species. The integration of CYP-dependent pathways and conjugation reactions in rat lung slices has been demonstrated for 7-ethoxyconuramin (35).

In this study lung slice viability was monitored to evaluate the culture conditions and to assess the effect of compound exposure on lung function. Protein synthesis demonstrated a constant and cumulative increase over the 48-hr culture period, indicating that the slices were viable under the culture conditions. Slice ATP levels, a marker of cellular energetics including the oxidation-reduction state of electron carriers, such as NADH and NAPDH, and for the ATP-producing machinery and functions, remained stable throughout the 48-hr culture period. Viability assessed by the leakage of low molecular weight DNA fragments into the medium also proved to be a valuable method which could be used for all eukaryotic cells.

This study has demonstrated that both human and rat lung are metabolically active toward cyclosporin derivatives. The biotransformation pathways for CSA and its hydroxethyl derivative IMM, which are known for liver, were present in the lung with hydroxylation at amino acid 1 to form AM1 or IMM1 as the major primary metabolite formed. AM1 formation from CSA is most likely to be cytochrome P450 dependent, as demonstrated by the partial inhibition with ketoconazole. In contrast, AM1 formation from SCP would be an esterase reaction occurring in the lung of both rat and human as well as in the liver. The lung will play an important role in the metabolism of each of these cyclosporin derivatives, particularly when administered via inhalation; however, the liver will be the major organ involved in the clearance of these compounds.

References


