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Cyclosporine Metabolites in Human Bile: Recovery and Immunologic Activity

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CYCLOSPORINE (CyA) is a highly metabolized cyclic peptide with potent immunosuppressive properties. Although the parent compound of CyA is considered to be primarily responsible for the immunosuppressive activity, initial observations with purified metabolite obtained by synthesis or by recovery from animal studies suggests that metabolites M17, M1, and M21 have immunosuppressant activity *in vitro*.¹ Human bile contains multiple CyA metabolites,² and the contribution of its metabolites to the overall immunosuppressive effect of the drug is as yet unknown. The objective of this study was therefore to (1) recover multiple fractions from human bile and (2) test their inhibitory effect on the growth of human lymphocytes *in vitro*.

MATERIALS AND METHODS

Metabolite Recovery

Pooled bile was collected from the T tubes of adult patients who had undergone orthotopic liver transplantation. A fresh 50-mL aliquot of bile was extracted with ethyl ether in a separatory funnel, the ether layer was obtained, and ether was removed under reduced pressure. The residue was reconstituted with aqueous methanol and washed in a separatory funnel with *n*-hexane, and the hexane layer was discarded. The hexane wash was repeated, and the aqueous methanol was taken to dryness under reduced pressure.

The high-pressure liquid chromatographic (HPLC) system consisted of a gradient of acetonitrile:water pumped through a 15-cm, 5- μ m C18 column (Supelco,

Bellefonte, PA) that was heated to 70°C. UV detection was performed at 214-nm wavelength. The complex gradient was adjusted to give the optimal separation of the component peaks over a period of 118 minutes. The residue from the extraction was reconstituted with methanol, and the injection volume was adjusted to permit maximal injection with the best possible peak separation. Component peaks were manually collected, the acetonitrile was removed, and the aqueous portion was lyophilized. Dry material from matched peaks of multiple injections was combined, and the identity of the material was confirmed as previously reported.²

Lymphocyte Proliferation Studies

Human peripheral blood lymphocytes were isolated from heparinized blood by the Ficoll-Hypaque technique. The lymphocytes (10^5 /well) were incubated at 37°C in 200 μ L tissue culture medium (TCM) supplemented with 10% human serum in the presence of concanavalin A (Con A, 10 μ g/mL) for 72 hours. During the final 20 hours of incubation, each culture was labeled with 1 μ Ci ³H-thymidine. The cultures were harvested and counted in a liquid scintillation counter as previously described. Unidirectional human mixed lymphocyte reaction (MLR) cultures were set up with 10^5 irradiated (2,000 R) stimulator cells in a volume of 200 μ L TCM supplemented with 10% human serum for six days. Proliferation was assessed by the degree of ³H-thymidine incorporation during the final 20 hours of incubation. The inhibitory effects of CyA and bile-derived metabolite fractions on Con A and MLR reactivity were measured at concentrations ranging from 0.005 to 5.0 μ g/mL. The results are expressed as percent inhibition or $(1 - [\text{cpm drug/cpm control}]) \times 100$.

RESULTS

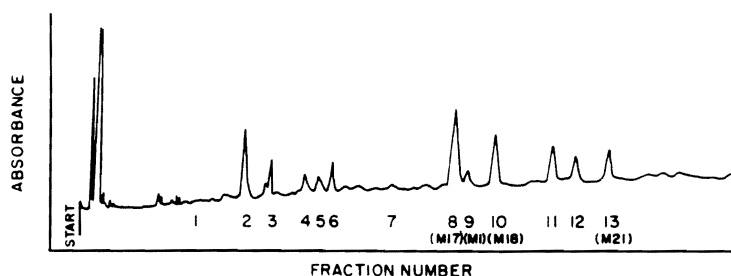
Thirteen major fractions were collected from the HPLC column, lyophilized, and combined with matched peaks to yield dry weights of between 0.65 and 2.41 mg. Figure 1 demonstrates an HPLC chromatogram of the injection of bile extract, the identification of each fraction on the chromatogram, and the corresponding identity of the peaks in relation to the nomenclature of Maurer.⁴ After identification, further HPLC analysis demonstrated

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Fig 1. An HPLC chromatogram of an injection of bile extract. Collected fractions are shown as are corresponding CyA metabolite designations.



that bile-derived M1, M18, and M21 were >95% pure by peak areas, whereas bile-derived M17 contained approximately 10% of an unidentified compound. The 13 components, including bile-derived M17, M1, M18, and M21, were subjected to immunologic testing.

In the test of inhibition with Con A-induced proliferation of peripheral blood lymphocytes, greater than 80% inhibition was observed with CyA concentrations of 0.5 $\mu\text{g}/\text{mL}$ or greater. Bile-derived M17, M1, and M21 produced approximately 60%, 50%, and 30% inhibition at the highest concentration (5 $\mu\text{g}/\text{mL}$), but no effect was noted in any of these fractions at 0.05 $\mu\text{g}/\text{mL}$ or lower. The same level of 50% inhibition of the Con A-stimulated response was observed with CyA in a 100-fold lower concentration (0.05 $\mu\text{g}/\text{mL}$). Figure 2 shows the results of the MLR studies. Greater than 80% inhibition of the MLR was observed for CyA at concentrations as low as 0.05 $\mu\text{g}/\text{mL}$. Significant inhibition was

observed with fraction 4 (unidentified) and with bile-derived M13, M17, M1, and M21 at the highest (5.0 $\mu\text{g}/\text{mL}$) concentration. Bile-derived M17 exhibited inhibitory action in the MLR similar to CyA even after the tenfold and 1,000-fold dilution.

DISCUSSION

CyA metabolites can be recovered from human bile and do have pharmacological activity when tested against *in vitro* lymphocyte cultures. Human bile contains many CyA metabolites, a number of which are only now being identified.⁵ Con A stimulation of peripheral blood lymphocytes was inhibited by several bile-derived metabolite fractions, with relative potency being CyA > M17 > M1 > M21 > M18. Although five bile fractions produced 40% or greater inhibition of the MLR in the 5.0 $\mu\text{g}/\text{mL}$ concentration, the activity of M17 was particularly striking in that it paralleled the activity of CyA even in 1,000-fold dilution. This observation is partic-

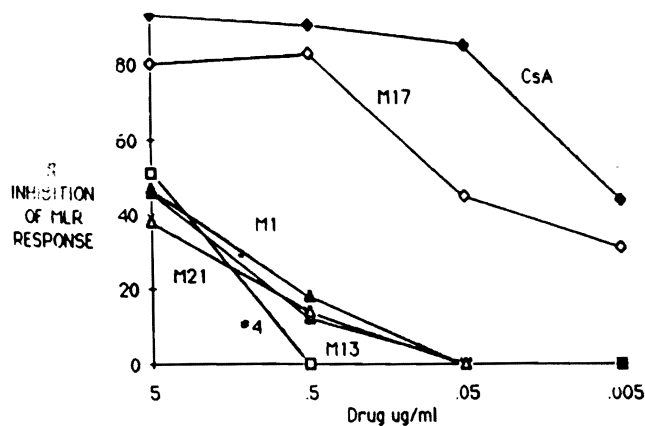


Fig 2. Percent inhibition of an MLR v concentration of CyA, its metabolites, and a fraction (*4) from the HPLC collection.

ularly important in that M17 frequently is found in the blood of transplant patients in equal or greater concentrations than CyA. Although the exact contribution of the metab-

olites to overall immunosuppressant activity is unclear, future studies of the action of CyA must take into account the presence of M17 and other metabolites.

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