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Rifampicin Inhibition of Protein Synthesis in Mammalian Cells

Abstract. *Rifampicin produces a dose-dependent decrease in protein synthesis in rat thymocytes. At concentrations up to 200 micrograms per milliliter, rifampicin does not alter rat thymic transcription. Rifampicin causes a direct inhibition of protein synthesis in rat thymic and hepatic microsomes, and in cadaveric human hepatic microsomes. Protein synthesis inhibition could explain the toxicity of rifampicin in man.*

The rifamycin antibodies have become important therapeutic agents since their isolation in 1959 (*1, 2*). Rifamycin binding to the β subunit of bacterial RNA polymerase results in the inhibition of transcription initiation (*3*). In contrast, eukaryotic transcription is not affected by rifampicin until much higher concentrations are attained (*4*). Clinical treatment of tuberculosis with rifampicin requires the oral administration of 300 to 600 mg/day (*5*). Oral administration of 600 mg of rifampicin per day to seven patients for 4 months resulted in mean peak serum concentrations of 7 μg/ml, whereas serum concentrations after a single 450-mg dose ranged up to 28 μg/ml (*1*). Organs such as the kidney, liver, and lung contained higher rifampicin concentrations than serum. After a single rifampicin dose of 450 mg, the liver contained 36 μg/g and bile 538 μg/ml (*1*).

Long-term administration of rifampicin has been shown to shorten the biological half-life of anticoagulants, glucocorticoids, oral contraceptives, and rifampicin itself, presumably through induced alterations in heptic metabolism (*6*). Rifampicin may induce abnormalities in heptic function, a toxic effect which may progress to necrosis (*5, 7*). Rifampicin is also immunosuppressive. In animals and in man, rifampicin inhibits both in vivo and in vitro humoral and cellular immunological responses (*8–10*). In man, rifampicin reduces lymphocyte proliferative responses in vivo to phytohemagglutinin and purified protein derivative (*9, 11*). Rifampicin applied locally to smallpox vaccination sites impaired the production of antibody (*12*). The formation of T lymphocyte rosettes and the response of humoral antibody to keyhole limpet hemocyanin were reduced in patients receiving rifampicin (*10, 13*).

In this report we demonstrate the effects of rifampicin on macromolecular synthesis in rat thymocyte suspensions in vitro. The most pronounced effect of rifampicin is a dose-dependent decrease in protein synthesis that begins at concentrations achieved in the serum of humans being treated with rifampicin. Protein synthesis is inhibited in the presence of increases in the specific activity of the soluble amino acid pool. Inhibition of protein synthesis by rifampicin is directly demonstrable in subcellular microsomal fractions from rat thymocytes and hepatocytes, and cadaveric human hepatocytes.

Male Simonsen Sprague-Dawley rats (60 to 100 g) were decapitated and their thymus glands excised, weighed, and minced in cold buffer containing 5.5 m*M* glucose, 5.0 m*M* KCl, 1.0 m*M* $MgSO_4$, 5.0 m*M* Na_2HPO_4, 120.0 m*M* NaCl, 5.0 m*M* tris, and 1.0 m*M* $CaCl_2$, adjusted to *p*H 7.2 at 25°C (*14*). Minced thymic tissue (0.05 g of thymus per milliliter of buffer) was transferred to a Dounce homogenizer and a cell suspension prepared with three strokes of a loose-fitting pestle. Cell suspensions were filtered, counted with a hemacytometer, and diluted to 170,000 thymocytes per milliliter of buffer. Thymocytes were then incubated with and without rifampicin under conditions which measured macromolecular synthesis. Rifampicin (500 μg/ml) was dissolved in buffer by stirring overnight at 2°C.

Rifampicin induced dose-dependent decreases in [^{3}H]thymidine, [^{3}H]uridine, and L-[^{3}H]leucine incorporation (Fig. 1). Similar decreases in protein precursor incorporation occurred when proteins were labeled with mixed ^{3}H-labeled L-amino acids in place of L-[^{3}H]leucine. The specific activity of the amino acid pool increases as the rifampicin concentration is increased (see Fig. 2). The data in Figs. 1 and 2 suggest that rifampicin inhibits protein synthesis in mammalian

Table 1. Effect of rifampicin on thymocyte transcription. Assays for RNA polymerase activity were performed according to Chamberlin and Berg (*20*) as modified by Roeder and Rutter (*21*). The assay medium contained 10 μmole of tris (*p*H 7.9), 0.5 μmole of 2-mercaptoethanol, 1.0 μmole of $MgCl_2$, 0.5 μmole of $MnCl_2$, 0.1 μmole of ATP, cytidine triphosphate (CTP), and GTP, and 0.01 μmole of uridine triphosphate (UTP). Tenfold increases or decreases in UTP concentration at constant specific activity indicated that the UTP concentration was not limiting. Calf thymus DNA was added in four- to fivefold excess (50 μg), then crude nuclear extract containing RNA polymerase was added to give a total of 150 μg of protein. The added nuclear extract containing RNA polymerase activity resulted in a final glycerol concentration of 3 to 5 percent. Maximum incorporations [expressed as disintegrations per minute (dpm) per assay] were determined in the presence and absence of rifampicin by adding 5 μCi of [5-^{3}H]uridine-5′-triphosphate (24 Ci/mmole) to initiate 15-minute periods of incubation at 37°C. Maximum incorporations were equal to incorporation rates at 8 minutes, after which incorporation plateaued unless higher glycerol concentrations were used (*22*). Transcription was terminated by the addition of cold $Na_4P_2O_7$ (100 μmole/ml) containing BSA (1 mg/ml), followed by 12 percent TCA. Precipitated material was collected by centrifugation at 17,300g_{max} for 10 minutes. Pellets were washed twice by dissolving in NaOH, then reprecipitated and prepared for counting as described for thymocytes (Fig. 1). Values shown are means (± standard error) of six replicates. All values are corrected for background counts and for color quenching. Average counting efficiency was 30 percent.

Rifampicin (μg/ml)	[^{3}H]UMP incorporation (dpm/assay)	
	Experiment 1	Experiment 2
0	1070 ± 48	1310 ± 70
20	1099 ± 60	1344 ± 70
200	1000 ± 128	1173 ± 117

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thymocytes. The incorporation of protein precursor appeared to decrease in the presence of increased specific activity of precursor, indicating that decreases in the incorporation of ^{3}H-labeled amino acids do, in fact, measure actual decreases in protein synthesis. Increases in soluble protein precursor pools have been observed previously in the presence of protein synthesis inhibitors (*15*).

To determine the effects of rifampicin on thymocyte transcription, we isolated

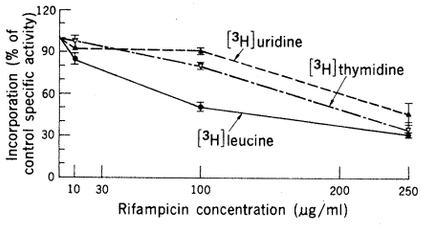

Fig. 1. Effects of rifampicin on incorporation of precursors of DNA, RNA, and protein into rat thymocytes in vitro. Glucose-tris buffer, rifampicin (0 to 250 μg/ml), and 1.9 μCi of [5-^{3}H]uridine (27.9 Ci/mmole), methyl-[H^3]thymidine (19 Ci/mmole) or L-[4,5-H^3]leucine (58 Ci/mmole) were added to assay tubes and the mixtures heated in a Dubnoff shaking incubator at 37°C for 5 minutes. Thymocytes (8500 cells in 0.05 ml) were then added to initiate incorporation for 10-minute periods at 37°C. These 10-minute periods were well within the linear incorporation period for all precursors studied. The total volume of individual assays was 1.0 ml. Incorporation was quenched by adding 1.0 ml of cold 12 percent trichloroacetic acid (TCA), followed by 5 ml of 6 percent TCA. Tubes were permitted to stand for 10 minutes in ice and then centrifuged for 10 minutes at 12,000 rev/min in a Sorvall SS-34 rotor (17,300g_{max}). Pellets were washed twice by dissolving the pellets in 1.0 ml of 0.1*N* NaOH, followed by reprecipitation in TCA. Washed pellets were drained and dissolved in 0.5 ml of tissue solubilizer and counted in 15 ml of toluene scintillation mixture [4.10 g of PPO and 0.1 g of POPOP per liter of toluene (*23*)]. Background counts caused by nonspecific binding of labeled precursors were estimated by incubating cells without label for 10 minutes, then adding label after the cells were cooled for 5 minutes at 0°C. These cells were then vortexed with label and immediately quenched. Samples containing rifampicin were orange-colored, and incorporations were also corrected by using the automatic external standard of a Packard 3320 TriCarb scintillation spectrometer and a color quench curve obtained by adding known amounts of label to experimental samples containing various concentrations of rifampicin. Specific activities were calculated, that is, disintegrations per minute of (i) [H^3]thymidine per milligram of DNA, (ii) [H^3]uridine per milligram of RNA, and (iii) [H^3]leucine per milligram of protein; these values were then plotted as percentages of control values. The DNA, RNA, and protein were chemically separated for analysis as follows. Cold 6 percent TCA (2 ml) was added to 0.2-ml portions of thymocytes and the samples were centrifuged for 10 minutes at 17,300g_{max}. The pellets were washed a second time according to the same procedure. The pellets were then resuspended in 1.0 ml of 6 percent TCA and heated at 70°C for 20 minutes. The samples were cooled at 0°C and centrifuged at 17,300g_{max} for 10 minutes. The DNA and RNA assays were performed on the supernatants, while the pellets were dissolved in 0.3 ml of 1.0 NaOH at 37°C for the measurement of protein. The DNA was determined by the method of Burton (*24*) with 2-deoxyribose being used as standard RNA by the orcinol method (*25*) with a ribose standard, and protein was determined by the procedure of Lowry *et al.* (*26*) with crystalline bovine serum albumin as standard. Each value shown is the mean ± standard error for 16 values from four separate experiments.

Table 2. Incorporation of ^{3}H-labeled amino acids by thymocyte and hepatic microsomal fractions in the presence and absence of rifampicin. Rat thymocyte microsomal fractions were prepared from 46 and 36 female rats (96 to 288 g), giving 70 and 100 μg of RNA per assay in experiments 1 and 2, respectively. Hepatic microsomal fractions were prepared from six female rats (114 to 246 g) giving 40 μg of RNA per assay in both experiments. Portions of microsomal fractions (0.05 ml) were incubated for 30 minutes at 37°C with 2 m*M* ATP, 0.25 m*M* GTP, 10 m*M* phosphoenolpyruvate, 50 μg of pyruvate-kinase per milliliter, 0.05 ml of cell sap or 0.025 ml of the *p*H 5 fraction, and 15 μCi of mixed ^{3}H-labeled amino acids (15 amino acids in proportions of algal hydrolysate) in a total volume of 0.2 ml with sucrose-tris buffer (*19*). Samples were quenched and prepared for counting as previously described. Figures shown are the average of two values, which always agreed within 5 percent. Corrections were made for background counts and color quenching. Average counting efficiency was 27 percent.

Rifampicin (μg/ml)	^{3}H Incorporation (dpm/assay)							
	Experiment 1				Experiment 2			
	Cell sap	% of control	*p*H 5 fraction	% of control	Cell sap	% of control	*p*H 5 fraction	% of control
				Rat thymocytes				
0	934	100	941	100	625	100	896	100
10	742	79	853	91	579	93	769	86
50	562	60	653	69	520	83	665	74
				Rat hepatic microsomes				
0	2643	100	3252	100	1481	100	1807	100
10	2316	88	3389	104	1381	93	1525	84
50	2272	86	2783	86	1508	102	1456	81

Table 3. Incorporation of ^{3}H-labeled amino acids by human hepatic microsomal fractions in the presence and absence of rifampicin. Cadaveric microsomal fractions were prepared from 30-g hepatic samples obtained from male and female trauma victims (3 to 69 years of age) less than 16 hours after death; the samples were kept frozen in liquid nitrogen. The fractions contained an average of 5.6 μg of RNA per assay. The other procedures were as described for Table 2, except that figures shown are the average of four values which always agreed within 5 percent. Average counting efficiency was 23.5 percent and was reduced to no more than 19.8 percent by the addition of 200 μg of rifampicin per milliliter.

Rifampicin (μg/ml)	^{3}H Incorporation (dpm/assay)							
	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Cell sap	% of control	Cell sap	% of control	Cell sap	% of control	Cell sap	% of control
0	1876	100	2311	100	1450	100	2159	100
20	1160	62	1760	76	1105	76	1808	84
50	1030	55	1495	65	991	68	1626	75
100	986	53	1450	63	958	66	1334	62
200	565	30	672	29	500	34	1219	56

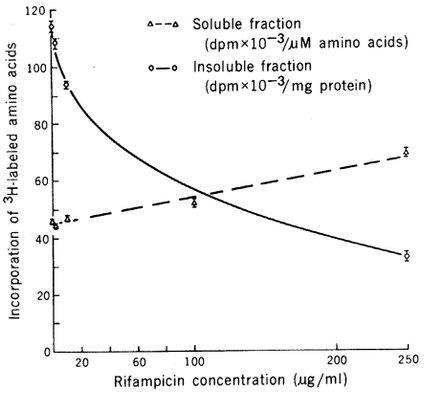

Fig. 2. Effect of rifampicin on the specific activities of ^{3}H-labeled amino acids in thymocyte soluble and insoluble fractions. The procedure was as described in Fig. 1 but with a twofold scale-up. A mixture of 15 ^{3}H-labeled L-amino acids in the proportion of algal protein hydrolysate (5 μCi per assay) was used in place of L-[^{3}H]leucine. After the thymocytes were incubated for 10 minutes in the presence of label, assay tubes were transferred to an ice-water slurry, and 5 ml of cold glucose-tris buffer was added. The cells were centrifuged for 10 minutes at 800 rev/min ($77g_{max}$) and the supernatant was carefully removed by aspiration. The cells were then washed twice by gently resuspending the pellets in 7.0 ml of cold buffer followed by recentrifugation. After the supernatant from the final wash was discarded, 5.0 ml of 6 percent TCA was added to the cell pellet. The denatured cells were then vortexed vigorously, allowed to stand in ice for 10 minutes, then centrifuged at $17,300g_{max}$ for 10 minutes. The supernatant was carefully removed and 0.5 ml was counted in 5.0 ml of Aquasol. Portions of the soluble fraction were also assayed for total amino acids according to a ninhydrin procedure (*27*). Values shown are specific activities (total disintegrations per minute $\times 10^{-3}$ from ^{3}H-labeled amino acids per micromole of total amino acids per assay). The insoluble fractions were solubilized for counting and plotted as total disintegrations per minute $\times 10^{-3}$ per milligram of protein (each assay contained 1.72 mg of protein). Figures shown are the mean ± standard error for four assays. All values are corrected for background counts and for color quenching. Average counting efficiency was 30 percent.

crude fractions of thymic nuclear RNA polymerase and used these to transcribe both calf thymus DNA and rat thymus chromatin in the presence and absence of rifampicin. To prepare thymic nuclear RNA polymerase and chromatin templates, we isolated nuclei according to the technique of Blobel and Potter (*16*). Purified nuclei were lysed with 15 strokes of a Teflon pestle in 0.05*M* tris buffer containing 0.005*M* dithiothreitol (DTT), 0.025*M* KCl, and 0.1*M* NH_4Cl, adjusted to *p*H 8.0 with concentrated HCl (DTT-tris). The nuclear homogenate was then incubated for 10 minutes at 25°C, a procedure resulting in the solubilization of 70 percent of the original nuclear RNA polymerase activity (*17*). Glycerol was added to a final concentration of 16 percent (weight to volume), and the nuclear lysate was centrifuged for 30 minutes at 39,000 rev/min ($269,000g_{max}$) in a Beckman SW 41Ti swinging bucket rotor at a temperature of 0°C. The resultant supernatant contained a high level of RNA polymerase II activity and was referred to as the crude nuclear extract. Chromatin was purified from the pellets according to the procedure of Huang and Huang (*18*) and contained no residual endogenous RNA polymerase activity. Table 1 shows that rifampicin had no significant effect on transcription in vitro when a crude thymic RNA polymerase fraction and calf thymus DNA were used as template. Experiments in which rat thymic chromatin was used gave similar results (data not shown).

To determine the direct effect of rifampicin on microsomal protein synthesis, we prepared rat hepatic and thymic and human hepatic microsomal fractions (*19*). Microsomes were then exposed to labeled amino acids in the presence and absence of rifampicin. A direct dose-dependent decrease in protein synthesis occurred in thymic microsomal fractions in the presence of rifampicin (see Table 2), and this inhibition was roughly commensurate with the degree of protein synthesis inhibition in whole thymocytes (Fig. 1). Inhibition of protein synthesis also occurred in hepatic microsomal fractions (Table 2), but was less pronounced than that in thymic microsomal fractions. Rifampicin produced a consistent decrease in protein synthesis in human microsomes (Table 3) that was significant at concentrations equivalent to those achieved in patients during therapy. Protein synthesis inhibition by rifampicin was more pronounced in human microsomes than in rat microsomes.

Our data indicate that rifampicin causes a direct inhibition of protein synthesis, and that this inhibition is pronounced at rifampicin concentrations equivalent to those achieved therapeutically. We propose that this effect may account, at least in part, for the immunosuppressive effects of rifampicin observed in man. Our finding that hepatic protein synthesis is inhibited by rifampicin suggests that this effect may account for the hepatitis or hepatic necrosis that is observed in some patients being treated with rifampicin. Protein synthesis inhibition may explain the inhibitory activity of rifamycin derivatives on tumor cell growth. The effects of rifampicin on thymocytes, along with data from clinical use of rifampicin, suggest that this antibiotic may be of therapeutic value for clinical immunosuppression in the treatment of immunologic disorders or following organ transplantation.

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