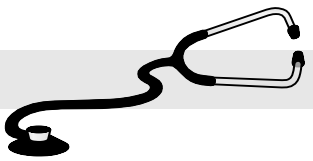

Rifampin-Induced Thrombocytopenia: Diagnosis by a Novel *In Vitro* Lymphocyte Toxicity Assay

Norberto Krivoy MD[1,2], Lili Struminger MSc[3], Regina Bendersky MD[2], Irit Avivi MD[4], Manuela Neuman PhD[5] and Shimon Pollack MD[3]

[1]Clinical Pharmacology Unit, [2]Department of Medicine B, [3]Institute of Immunology and Allergy and [4]Division of Hematology, Rambam Medical Center and Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, Haifa, Israel; and [5]In-Vitro Toxicology Laboratory, Sunnybrook HSC, Toronto, Ontario, Canada

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Several cases of rifampin-induced thrombocytopenia have been reported in the literature [1]. It was recently demonstrated that many of the rifampin-induced adverse events have an allergic origin. While non-reaginic anti-rifampin antibodies were detected in the serum of patients, anaphylactic reactions due to the use of the drug seem to be immunoglobulin E mediated [2].

Re-challenge and de-challenge of a drug are established tools but not accepted techniques for the diagnosis of adverse drug events. We report here the case of a woman who developed severe thrombocytopenia, hemolytic anemia and high serum levels of IgE, without renal function impairment. Her condition was evaluated by use of a modified *in vitro* lymphocyte toxicity assay.

Patient Description

A 40 year old woman (47 kg weight) of Ethiopian origin was admitted for the investigation of fever (38°C) and dry cough that had persisted for the previous 2 weeks. Her past history was unremarkable and she was not taking any fixed medications. On physical examination her body temperature was 38.2°C and she had diffuse wheezing and dry crackles in the lungs. The relevant laboratory findings are shown in Table 1. Anti-hepatitis B and C antibodies were found. Hepatitis B surface antigen was not detected. An enzyme-linked immunosorbent assay test for human immunodeficiency virus was negative, as was the viral load test.

A chest X-ray on admission demonstrated alveolar infiltration in the left and right lungs, a finding compatible with an infective process. A purified protein derivative (1/100) skin test was negative. A sputum specimen revealed positive acid-fast bacilli, and a specific gene probe identified groups of *Mycobacterium tuberculosis hominis* bacteria. Isoniazide 300 mg/day, pyrazinamide 1,500 mg/day, ethambutol 1,200 mg/day and rifampin 600 mg/day were prescribed.

On the day that rifampin was discontinued a direct Coombs' test (anti-IgG positive, anti-C3 traces) was positive; slight erythrocyte fragments and polychromatophilia were observed in a peripheral blood smear. A qualitative test for urine hemoglobin was slightly positive. Normal numbers of megakaryocytes without the presence of abnormal cells were found in the bone marrow aspiration that was mixed with peripheral blood. A bone marrow biopsy performed on admission did not demonstrate granulomata and no abnormal cells were present. The histological specimen showed mild hypercellularity and a mild increment of megakaryocytes was found. The IgE serum concentration was 320 KU/ml (normal up to 183 KU/L).

Forty-five days after cessation of the rifampin therapy her platelet count rose to 98 x 10^3 ml. She was admitted to the emergency room because of acute gastroenteritis. Her blood count showed hemoglobin 13.4 g/dl, white blood cells 4.8 x 10^3 ml, platelets 189 x 10^3 ml, and mean corpuscular volume 87.0 fl.

An *in vitro* lymphocyte toxicity assay was performed to establish the possible relationship between the prescribed medications and the adverse events. A fresh whole-blood sample was layered on a Ficoll-Paque density gradient, and interphase cells were collected, layered on 20% sucrose and then suspended in a Hepes-buffered medium. The pellet was re-suspended to yield 10^6 cells/reaction (viability at this stage was 95%). Lymphocytes were collected by centrifugation, the supernatant was aspirated, and NADPH, G6P and G6PD (Sigma, USA) were added. The cells were counted and re-suspended again at a density of 2 x 10^6/ml in tubes. Untreated lymphocytes were considered as controls. The lymphocytes treated with different drugs without microsomes comprised the second set of controls, and lymphocytes re-suspended with drugs and canine micro-

IgE = immunoglobulin E

Table 1. Laboratory data

Date	Platelets ($x10^3$/ml)	Hb (gr/dl)	Retic (%)	MCV (fl) (80–94)	LDH (IU/L) (60–225)	GGT (IU/L) (5–60)	AST (IU/L) (5–40)	ALT (IU/L) (5–40)	AP (IU/L) (30–115)	Therapy
29 Feb	265	10.7		77.1						
2 Mar	274			79	224	21	19	15	51	
5 Mar	225									
6 Mar										Anti-tuberculous therapy**
11 Mar	12	8.4		79.4						Rifampin stopped
12 Mar	22	8.1	3.4	80.7						
13 Mar	10	8.3			271	22	18	10	57	Prednisone 40 mg/day, tranexamic acid 1 g/6 hr
15 Mar	88	10	6.8	82	287					IVIG 25 g/day
17 Mar	97	9.9								
19 Mar	1	9.3	5							
20 Mar	1	11.3	4.2		250	25	34	21	61	Last IVIG dose
24 Mar	5	9.2			230	21	33	23	42	
26 Mar	14	10.8	10.2	91.1						
27 Mar	16	9.3			270	56	145	116	55	

Figures in parenthesis represent normal values.
** Isoniazide, rifampin, ethambutol, pyrazynamide.
Retic = reticulocytes, LDH = lactic dehydrogenase, GGT = gammaglutamyl transferase, AST = aspartate aminotransferase, ALT = alanine aminotransferase, AP = alkaline phosphatase, IVIG = intravenous hyperimmune gammaglobulin.

somes (Promega, USA) were considered as the test cells. Each test was done in triplicate, and 24 hours later cells were washed and plated onto microplates at 37°C. Formazan (MTT, 100 µl) was added for 2 hours, the toxicity assay was performed and the results read by an ELISA reader at 595–650 nm. The percentage of cells displaying cytotoxicity, compared to control cells treated only with the drug, was calculated. The normal result for this test is <15% cytotoxicity, between 15% and 20% of cytotoxicity is undetermined, and above 20% is frankly positive. The cytoxicity results were 61% for rifampin and 15% for ethambutol. Pyrazynamide and isoniazide revealed no cytotoxic response. The control group was established to be 15% cytotoxicity.

ELISA = enzyme-linked immunosorbent assay

Comment

We present a woman who developed severe and persistent rifampin-induced thrombocytopenia, hemolytic anemia, slight intravascular hemolysis and high serum levels of IgE, without renal function impairment. The use of a modified *in vitro* lymphocyte toxicity assay allowed us to reach the diagnosis of this drug-induced cytotoxicity phenomenon, without performing the classical de- and re-challenge procedure.

It can be assumed that the mechanism/s of rifampin-induced thrombocytopenia and hemolysis are due to reactive metabolite/s and/or rifampin metabolites such as the desacetylrifampin and/or formylrifampin, which may covalently link to host blood cell proteins and form hapten-protein conjugates. The resulting anti-drug antibody damages the cell membrane. In addition, the aberrant expression of hapten-conjugate on cellular membranes in conjunction with major histocompatibility complex I molecules may lead to a cytotoxic response against the blood cells.

The resultant immune responses may give rise to rifampin-induced immune hemolytic anemia, intravascular hemolysis, hemoglobinuria and/or thrombocytopenia. The increased sensitivity to reactive metabolites of a drug, in particular those produced by microsomal oxidation, seems to be inherited. The presence of rifampin-dependent antibodies of the IgM class, which had anti-I specificity against erythrocytes and an IgG anti-rifampin antibody with complement-fixing capability, has been described [3].

Other possible mechanisms involved is the presence of a Fab terminal anti-

drug antibody that binds to the GPIb/IX or to the GPIIb/IIIa platelet receptor, causing drug-induced thrombocytopenia such as in quinidine-induced thrombocytopenia [4]. Our patient's delayed recovery from thrombocytopenia after discontinuation of the drug and the later reappearance of severe thrombocytopenia may be attributed to the generation of rifampin-associated IgG antibodies (PAIgG) [5].

The relevance of lymphocytotoxicity assay to those mechanisms is based on the reactive drug metabolite/s, which are generated *in vitro* using canine microsomes as a source of cytochrome P-450. Human lymphocytes from patients with suspected hypersensitivity reactions are used, as surrogate target cells form *in vitro* rechallenge. If the cells are susceptible to damage by reactive metabolites, the *in vitro* toxicity can be measured by using MTT, which is converted into a purple formazan by viable cellular mitochondrial succinate dehydrogenase.

Using this modified *in vitro* laboratory test, we demonstrated that a convincing 61% cytotoxicity developed after 5 days exposure to rifampin. The use of this novel *in vitro* lymphocyte cytotoxicity assay allowed the documentation of this specific drug-induced adverse event. In general, it is recommended that an accurate laboratory assay be used to establish the relationship between drugs and adverse events instead of using the "old" de-and re-challenge policy.

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Correspondence: Dr. N. Krivoy, Clinical Pharmacology Unit, Rambam Medical Center, Haifa31096, Israel. Phone: (972-4) 854-2676, Fax: (972-4) 854-3252,
email: N_Krivoy@rambam.health.gov.il