Absence from Sera from Normal Individuals or from Rifampin-treated Leprosy Patients (THELEP Trials) of Antibody to Rifamycin-protein or Rifamycin-membrane Conjugates[1]

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Treatment with rifampin, particularly if intermittent, can be associated with side effects which appear to have an immunological basis (see Dewdney[4] for review). The Scientific Working Group on the Chemotherapy of Leprosy (THELEP), UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, therefore decided to monitor the presence of rifampin-dependent antibodies in the serum of leprosy patients included in THELEP trials involving six rifampin-containing regimens (Table 1).

A number of techniques have been used to demonstrate rifampin-dependent antibodies. The indirect Coombs test can be used to demonstrate complement on the surface of compatible human red cells which have been incubated in positive donor serum in the presence of the drug ([2, 10]). This test gives a positive correlation with the occurrence of a "flu-like syndrome" ([15]) in rifampin-treated individuals but is negative with normal sera.

In sharp contrast, Stevens, *et al.* ([13]) have claimed to detect antibody using rifamycin/protein conjugates in a gel precipitation assay. Moreover, this antibody was said to exist in almost all sera, whether rifampin-treated or not, and to decrease rather than rise in those individuals developing clinical side effects ([13]) (perhaps due to consumption by immune complexes).

Therefore with the dual purpose of monitoring the THELEP patient groups and of casting light on the dilemma posed by the conflicting results of the two assays outlined above, we have measured antibody to rifamycin-protein and rifamycin-membrane conjugates using the enzyme-linked immunosorbent assay (ELISA), while aliquots of the same samples have been studied by the indirect Coombs test at the Hammersmith Hospital, London, in the department of the late Dr. S. Worlledge ([10]).

MATERIALS AND METHODS

Donors and serum samples. A total of 239 sera have been screened. Seventy-two sera from 36 patients were provided under the THELEP program by Dr. P. S. Seshadri of the Central Leprosy Teaching and Research Institute, Chingleput, India. All sera were sent frozen, on dry ice, until thawed and aliquotted in the laboratories of Dr. R. J. W. Rees (NIMR, Mill Hill, London, England). They were then re-frozen and stored at −26°C. Nine patients received regimen A_1, 12 received regimen C, and 12 received regimen D_1 (Table 1). From each patient a sample was taken before treatment and a further sample (or samples) was taken at an appropriate interval after treatment. For patients on regimen A, this was three months or more after commencement of therapy. For patients receiving regimen C or D, this was two weeks after the single initial dose.

A similar series of 53 sera from 20 patients was sent under the THELEP program by Dr. C. Ferracci, Institut Marchoux, Bamako, Mali. Post-treatment sera were not obtained from two patients. The remainder included 3 receiving regimen A_2 (post-treatment samples at 3, 6 or 12 months); 7 receiving regimen E_2 (post-treatment samples at 3 months when rifampin treatment ended, and then at 4 months and in 4 cases, at 6 months); 8 receiving regimen C (post-

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TABLE 1. *THELEP regimens.*

Regimen no.	Drug	Dose	Duration	Post-treatment serum samples
		Chingleput trial		
A_1	Dapsone	100 mg daily	Indefinitely	>3 months
	Rifampin	600 mg daily	Indefinitely	
	Clofazimine	100 mg daily	Indefinitely	
C	Dapsone	100 mg daily	Indefinitely	2 weeks
	Rifampin	1500 mg	Single initial dose	
	Placebo	One daily	First 3 months	
D_1	Dapsone	100 mg daily	Indefinitely	2 weeks
	Rifampin	1500 mg	Single initial dose	
	Clofazimine	100 mg daily	First 3 months	
		Bamako trial		
A_2	Dapsone	100 mg daily	Indefinitely	3, 6, or 12 months
	Rifampin	600 mg daily	Indefinitely	
	Prothionamide	500 mg daily	Indefinitely	
C	Dapsone	100 mg daily	Indefinitely	2 weeks and 1 month
	Rifampin	1500 mg	Single initial dose	
E_2	Dapsone	100 mg daily	Indefinitely	3, 4, and 6 months
	Rifampin	900 mg once weekly	First 3 months	
	Prothionamide	500 mg daily	First 3 months	

treatment samples at 2 weeks, and in 6 cases, 1 month after the single dose of rifampin).

The assays described below were also applied to sera in our own collection. These included 10 from normal Indians, 30 from normal Europeans, and 70 sera from leprosy patients under the care of Dr. Desikan and Dr. Ramu at the JALMA Institute, Agra, India. The majority of these patients had never received rifampin.

Four sera were provided by E. Lloyd from the Department of Hematology, Hammersmith Hospital, London. These sera were all positive by the indirect Coombs test technique for rifampin-dependent binding of complement to erythrocytes ([10]), and were derived from patients included in earlier studies from this laboratory ([10, 15]) who had developed the "flu-like" syndrome following twice weekly rifampin therapy.

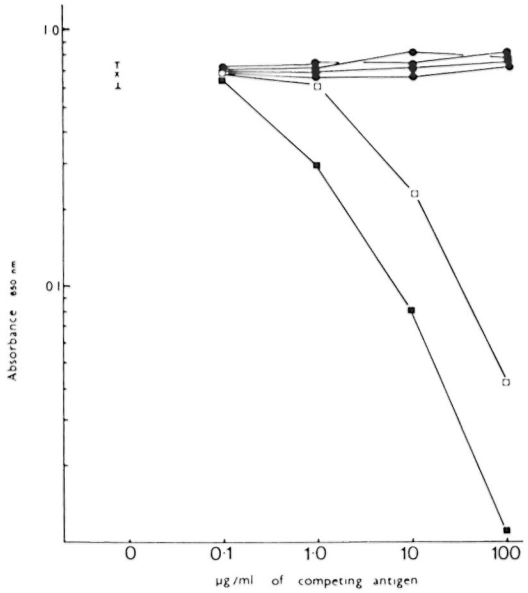
Reagents. 3-Formyl rifamycin and 1-formyl amino-4-methyl piperazine and rifampin were gifts from CIBA-GEIGY. Keyhold limpet hemocyanin was purchased from Calbiochem. Tween 20, human serum albumin, azobenzarsonate and horseradish peroxidase type VI were obtained from Sigma. Anti-sera to mouse Ig and the human α, γ and μ chains were obtained from Dako, and conjugated to peroxidase by the method of Nakane and Kawaoi ([9]).

Preparation of rifampin-conjugated proteins. Ten mg of 3-formyl rifamycin was mixed with 16 mg of human serum albumin or keyhole limpet hemocyanin in 4 ml of 0.1 M carbonate/bicarbonate buffer, pH 9.6, for 2 hr. The resulting Schiff bases were extensively dialyzed against phosphate-buffered saline and stored at 4°C with sodium azide. The resulting conjugates contained approximately 20 molecules of rifamycin for each molecule of protein ([14]).

Preparation of control anti-sera. Groups of BALB/c mice were immunized with 50 μg of the rifamycin-conjugated products in 0.2 ml of incomplete Freund's adjuvent divided into four sites. The immunization was repeated at one month, and the animals were bled 14 days later. The conjugates evoked good anti-sera (see Results).

Attempts were also made to raise an antibody to 1-formyl amino-4-methyl piperazine, using the same conjugation procedure and immunization schedule.

Control antigens. Dapsone and azobenzarsonate were diazotized and conjugated



THE FIGURE. Specificity of the mouse anti-rifamycin. All wells were coated with RIF-HSA. Absorbance values indicate the binding of the mouse anti-RIF-KLH to this antigen in the absence of competing antigen (×) (range indicated) or in the presence of increasing concentrations of RIF-HSA (■), rifampin (□), or HSA, dapsone conjugated HSA, or azobenzarsonate conjugated HSA (●).

to human serum albumin as described by Huikeshoven [7]. When used for specificity control in blocking experiments, these antigens were diluted in phosphate-buffered saline containing 0.05% Tween 20 and added to the wells immediately before the addition of the antiserum.

Coating microtiter wells with lymphocyte or erythrocyte membranes. Human tonsillar lymphocyte membranes were a gift from Dr. P. M. Lydyard. They had been prepared by the method of Crumpton and Snary [3]. Human group 0, Rhesus negative erythrocyte membranes were prepared as described by Dodge, Mitchell and Hanahan [5].

Wells were coated with membranes as described elsewhere [11]. The membranes on some wells were then conjugated with 3-formyl rifamycin, while others served as controls for anti-erythrocyte or anti-lymphocyte antibodies. Conjugation was achieved by the addition to membrane-coated wells of 0.2 ml of a solution of 3-formyl rifamycin (1 mg/ml) in 0.1 carbonate/bicarbonate buffer, pH 9.6. After incubation at room temperature for 1 hr, the wells were extensively washed, and the standard ELISA protocol followed.

Enzyme-linked immunosorbent assay. Human sera were diluted to 1/200 in phosphate-buffered saline containing 0.05% Tween 20, and assayed as described elsewhere [11]. Protein conjugates were coated onto the wells at 2 μg/ml. Since the rifamycin was conjugated to proteins or membranes, control wells coated with the unconjugated carrier were always included. Pilot experiments showed that testing the sera at higher concentrations increased the binding to the carrier without significantly altering the ratio of binding to carrier relative to conjugate.

Coombs test for rifampin-dependent antibody. An aliquot of each of the sera from the THELEP trial was studied by the Coombs test [10] at the Hammersmith Hospital, London.

Clinical observations. No "flu-like" syndromes attributable to reactions to rifampin were reported by the clinicians involved. Four patients from Bamako became jaundiced during treatment. Their sera were screened for hepatitis B and A by Dr. D. Dane (Department of Microbiology, Middlesex Hospital Medical School, London). No explanation for the jaundice was found.

RESULTS

Establishment of positive control sera. The antibody raised in BALB/c mice against rifamycin-keyhole limpet hemocyanin (RIF-KLH) was assayed against wells coated with human serum albumin (HSA) or rifamycin/human serum albumin conjugate (RIF-HSA). This binding was blocked in a dose-related manner by simultaneous addition of rifampin or rifamycin/HSA, but not by HSA, dapsone, dapsone/HSA or arsanilate/HSA. This antibody was therefore used as a positive control for antibody to rifamycin (The Figure).

However, we were unable to demonstrate antibody to 1-formyl amino-4-methyl piperazine in sera from mice immunized with this compound conjugated to protein carriers. Thus we did not have a positive control for antibody to the side-chain of the rifampin molecule.

Results with human sera using rifamycin/HSA as antigen. All the human sera were screened at a dilution of 1:200 for

TABLE 2. *Reactivity of positive control sera for IgM binding.*

Antigen	Absorbance values	
	Human serum[a]	Mouse anti-rifamycin
Human serum albumin (HSA)	0.01	0.02
RIF[b]-HSA	0.02	0.24
Human tonsillar lymphocyte membranes (HTLM)	0.06	0.03
RIF-HTLM	1.30	0.28
Human erythrocyte membranes	0.02	Not done
RIF-erythrocyte membranes	0.70	Not done

[a] Serum known to be positive for rifampin-dependent antibodies by the Coombs test. Both sera used at 1:200.

[b] Rifamycin conjugated.

binding of IgG, IgA, or IgM to rifamycin/HSA. In no case was the binding to rifamycin/HSA significantly greater than the binding to HSA alone. The four sera known to be positive by the indirect Coombs test, using the whole rifampin molecule, were also negative by this technique although the BALB/c anti-rifamycin serum was consistently strongly positive.

Reaction of positive control anti-sera with rifamycin membrane conjugates. The four control sera positive by the indirect Coombs test and the positive BALB/c serum were assayed using rifamycin-conjugated human tonsillar lymphocyte membranes and erythrocyte membranes. One of the four Coombs test positive sera gave positive results for IgM binding when either type of membrane was used as the carrier, but not when HSA was used (Table 2).

All of the sera were screened for IgM, IgG, and IgA antibodies to rifamycin-conjugated erythrocyte membranes. The sera discussed above (Table 2) were used as positive controls. Again, no sera, except the two controls, showed significantly increased binding to the conjugate. Similarly, there was no significantly increased binding if the post-treatment sera from the treated group most at risk (regimen E_2) were considered separately.

Coombs test. The sera were also all negative by the Coombs test (E. Lloyd, personal communication), although the technique used was as for previous studies from the Hammersmith Hospital ([10, 14]) and controls from these earlier studies were positive.

DISCUSSION

We have not detected antibody to rifamycin conjugates or rifampin-dependent Coombs test positivity in the sera of patients from the THELEP trials. The risk of the latter is greatest with large intermittent doses, especially if taken twice per week, or in individuals who have been on rifampin treatment and then resume taking the drug after an interval ([10, 15]). Therefore, those taking regimen E_2 were the most at risk but were still negative by all the assays, including the Coombs test. However, the number of patients in the group was clearly too small to allow any clear statement about the safety of regimen E_2. There were no reactions attributable to the drug regimens.

We have also failed to confirm the existence in most sera, whether from rifampin-treated individuals or not, of antibody to rifamycin-protein conjugates.

The ELISA has several advantages for this type of study. First, it is a binding assay and so it does not rely on secondary characteristics of the putative antibody, such as complement fixation as in the indirect Coombs test used by Worlledge and her colleagues, or precipitation, as in the work of Stevens, *et al.* Secondly, it is sensitive and allows separate assessment of the major antibody classes. Thirdly, it allows the use of a variety of different carrier molecules.

However, the ELISA using rifamycin-protein conjugates resulting from a Schiff base formation between 3-formyl rifamycin and amino groups (mostly the E-amino of lysine) would not be expected to detect the antibody revealed by the indirect Coombs test because analysis of the latter assay ([6]) suggests involvement of both the rifamycin nucleus and the hydrazone structure, which in rifampin links the rifamycin nucleus to the side chain. (The side chain itself, 1-amino-4-methyl piperazine, is inactive.) The

Schiff bases which we and Stevens have used do not contain the hydrazone. Moreover, not all authors are convinced that this Coombs test is due to a true antibody, and it is usually negative if anti α, γ or μ are used, rather than anti-complement ([12]). For these reasons the negativity of the four Coombs test positive controls in our system was anticipated.

We have also failed to confirm the assertion of Stevens, *et al.* that essentially all normal individuals have antibody which forms precipitates with rifamycin-conjugated proteins ([13]). A direct binding assay such as the ELISA will usually detect precipitating antibodies; however, an exception is possible. Moreover, in the absence of a positive human serum we have been forced to use a mouse serum as positive control for setting up the assay, so it may not be optimal for detection of the antibodies described by Stevens and his colleagues. Nevertheless, an alternative explanation is that the "antibodies" detected by these authors were artefacts due to the use of a low molarity buffer (0.03 M PBS) and 3.25% polyethylene glycol (L. Bassi, DOW, Italian Region, and Dr. F. Bloemmen, Department of Clinical Immunology, Pellenberg, Belgium, personal communications) which cause exaggerated precipitating conditions. This situation may have been aggravated by the fact that conjugation of proteins with rifamycin causes some aggregation and decreases solubility.

It is perhaps more surprising that antibody to rifamycin-serum-protein conjugates was not found in the serum of rifampin-treated patients. Part of an orally ingested dose of rifampin becomes converted to 3-formyl rifamycin because of the relative instability of the hydrazone bond. This product readily forms Schiff bases with the E-amino group of lysine, so rifamycin-protein conjugates inevitably form *in vivo*. It is possible, however, that these are very poorly immunogenic unless injected in complete Freund's adjuvant, as in the work with rabbits ([8]) or mice (present study).

Schiff base formation with membrane components, particularly with products of the major histocompatibility complex, would be more likely to yield products which were immunogenic without adjuvant. Nevertheless, no such antibodies were found, except in one serum known to contain rifampin-dependent antibody by the Coombs test. This serum contained an interesting antibody which bound to RIF-membranes but not to RIF-serum proteins (data not shown). This approach to the search for antibodies causing drug reactions clearly merits further study.

SUMMARY

It has been reported that normal individuals have precipitating antibody which binds to rifamycin-conjugated proteins. An enzyme-linked immunosorbent assay has failed to confirm this claim, although antibodies demonstrable in a solid-phase binding assay are easily raised in mice if complete adjuvant is used. Moreover, no antibodies to rifamycin-protein conjugates were found in sera from the patients included in THELEP trials of six rifampin-containing regimens. Similarly, there was no antibody by the indirect Coombs test performed in another laboratory.

Further studies using rifamycin-membrane conjugates regarded as more likely to be immunogenic *in vivo* also failed to reveal antibody in patients' sera, although this technique revealed an interesting antibody in one of four control sera known to be positive by the indirect Coombs test.

RESUMEN

Se ha publicado que los individuos normales tienen anticuerpos precipitantes que reaccionan con rifamicina conjugada a proteínas. Nosotros no pudimos confirmar ésto usando un enzimoensayo pero encontramos que tales anticuerpos son fácilmente inducibles en ratones si se usa adyuvante completo. Tampoco pudimos encontrar anticuerpos contra rifamicina en los sueros de los pacientes tratados en los ensayos del THELEP con seis esquemas de tratamiento donde se incluia rifampina. De igual manera, no se encontraron anticuerpos por la prueba indirecta de Coombs realizada en otro laboratorio.

Estudios posteriores usando conjugados de membrana-rifamicina (considerados como posiblemente más inmunogénicos *in vivo*) también han resultado fallidos en cuanto a la demostración de anticuerpos en los sueros de los pacientes, sin embargo, ésta técnica permitió descubrir un anticuerpo interesante en uno de cuatro sueros control que habían resultado positivos por la prueba de Coombs indirecta.

RÉSUMÉ

On a relaté que des individus normaux possèdent des anticorps de précipitation qui peuvent être liés aux

protéines conjuguées à la rifamycine. Une épreuve ELISA n'a pas permis de vérifier cette observation. Il est cependant facile d'induire chez la souris des anticorps qui peuvent être mis en évidence dans une épreuve en phase solide, lorsqu'on utilise un adjuvant complet. De plus, aucun anticorps ou conjugués rifamycine-protéine n'a été observé dans le sérum de malades qui participaient aux essais THELEP portant sur six schémas thérapeutiques différents comprenant de la rifampine. De même, aucun anticorps n'a été décelé au moyen d'une épreuve indirecte de Coombs, menée dans un autre laboratoire.

Des études complémentaires faisant appel aux conjugués rifamycine-membrane, considérés comme beaucoup plus propres à démontrer un effet immunogénique *in vivo*, ont permis de mettre en évidence des anticorps dans le sérum des malades. Cette technique a cependant permis de déceler un anticorps intéressant dans un échantillon de sérum, parmi des échantillons provenant de quatre malades dont on savait qu'ils étaient positifs à l'épreuve indirecte de Coombs.

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