Evidence for immunological (allergic) mechanisms in a subgroup of patients with phenprocoumon-induced liver disease

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PHARMACOKINETICS AND DISPOSITION

Evidence for immunological (allergic) mechanisms in a subgroup of patients with phenprocoumon-induced liver disease

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Abstract
Purpose Phenprocoumon-induced liver injury is a rare complication of oral anticoagulation. The mechanisms leading to this side effect are not entirely clear. Here we present data that at least in a subgroup of patients in whom phenprocoumon-induced liver disease was suspected, immunological processes may play an important role.
Patients and methods Thirty patients with suspected phenprocoumon-induced liver disease from different hospitals in Germany were analyzed. Peripheral blood mononuclear cells (PBMC) of these patients were tested in the lymphocyte transformation test (LTT) for reactivity with phenprocoumon in vitro. As controls, PBMC were isolated from ten individuals treated with phenprocoumon but without any side effects, and from ten healthy individuals who have never received the drug.
Results Fifteen of the 30 patients had sensitized lymphocytes toward phenprocoumon as shown by LTT. Four patients had taken the drug for more than 5 years; in one patient, liver disease appeared after 1 day of phenprocoumon intake. There was no correlation between a positive LTT and clinical/laboratory parameters. None of the 20 controls had sensitized lymphocytes toward phenprocoumon.
Conclusions Applying the LTT, we were able to unravel the cause of suspected phenprocoumon-induced liver injury as a drug allergic reaction in 15 out of 30 analyzed patients.

Keywords Allergy · Drug-induced liver injury · Lymphocyte transformation test · Phenprocoumon

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Abbreviations

ALAT	Alanine aminotransferase
ANCA	Antineutrophil cytoplasmic antibodies
AP	Alkaline phosphatase
ASAT	Aspartate aminotransferase
CD	Cluster of differentiation
Cpm	Counts per minute
CYP	Cytochrome P450
DILD	Drug-induced liver disease
IKS	Pharmacovigilance Centre
LTT	Lymphocyte transformation test
MPO	Myeloperoxidase
PBMC	Peripheral blood mononuclear cells
SANZ	Swiss Drug Monitoring Centre
SI	Stimulation index
ULN	Upper limit of normal

Introduction

Liver injury as a side effect of oral anticoagulants—such as the coumarin derivatives phenprocoumon, Warfarin sodium, and acenocoumarol—has been only rarely reported worldwide [1], whereas most frequently, bleeding complications occur. The Swiss Drug Monitoring and the Pharmacovigilance Centers (SANZ and IKS) recounted ten (1.5%) oral-anticoagulant-related cases among 674 reports of drug-induced liver disease (DILD) between 1981 and 1995 [2]. In Germany, 0.89% of all reported drug-induced adverse reactions affecting the liver seem to be phenprocoumon related [1].

Clinically/histologically, phenprocoumon-induced liver disease ranges from mild acute hepatitis to (sub) acute liver failure [1, 3]. There are only a few reports about cholestatic

hepatitis [2, 4]. The mechanisms leading to phenprocoumon-induced liver injury are not entirely clear. There are three case reports suggesting immunological processes due to a positive liver transformation test (LTT) or the activation of CD4-positive memory T cells [5–7]. Also, eosinophilic infiltrates in liver biopsies of patients with phenprocoumon-induced liver disease would support the concept of an immunologically mediated process, although eosinophilia in the blood or clinical signs of allergy (exanthema, fever) has not been observed [1, 8, 9].

Over the past 20 years, we collected a series of 30 patients with DILD in whom phenprocoumon was highly suggestive to be the causative agent. We show that 15 of them had sensitized lymphocytes toward this substance, indicating that at least in a subgroup of patients, immunological/allergic processes may, indeed, play a role in the pathogenesis of the liver injury.

Patients and methods

Patients

In the period 1997−2007, we received peripheral blood from 204 patients in whom a drug-induced allergic liver disorder had been suspected; 51 of them (25%) had taken coumarin derivatives. Twenty-one patients had to be excluded from the study due to the lack of detailed clinical and biochemical data, i.e., 30 patients were further analyzed. In all of them, lymphocyte transformation test (LTT) had been performed in the optimal time period after withdrawal of the drug, which ranged between ^{2}10 and 48 days [11, 12]. Four patients had already taken the drug for more than 5 years. In the remaining 26 patients, the median drug intake until the appearance of DILD was 6 months. There was only one patient in whom features of the injury occurred 24 h after first intake of the drug. In seven of the 30 patients, the drug had not yet been withdrawn at the time of LTT.

Reasons for phenprocoumon intake were atrial fibrillation (60%), mitral valve replacement (16%), and coronary heart disease (8%). Two further patients received phenprocoumon because of several thromboembolic events; one of them suffered from vasculitis with antibodies to neutrophils/myeloperoxidase (pANCA/MPO). Eighteen patients took other drugs besides phenprocoumon.

The diagnosis of DILD was based on the following International Consensus Meeting criteria [12]: (1) alanine aminotransferase (ALAT) > 2 times the upper limit of normal (ULN); conjugated bilirubin > 2 times ULN; or combined increase in aspartate aminotransferase (ASAT), alkaline phosphatase (AP), and total bilirubin provided one was > 2 times ULN; (2) identification of a medicinal agent (in this study, phenprocoumon) with a temporal relationship to elevated liver enzymes; and (3) exclusion of other causes of liver or biliary-tract disease and excessive use of alcohol.

International Consensus Criteria were used to define the pattern of liver injury as hepatocellular, cholestatic, or mixed [13]:

- Hepatocellular: ALAT ≥ 2 times ULN, or R ≥ 5 (R = ALAT/AP)
- Cholestatic: AP ≥ 2 times ULN or R ≤ 2
- Mixed: ALAT ≥ 2 times ULN and AP ≥ 2 times ULN and 2 < R < 5.

Liver biopsy had been performed in only two of the 30 patients with respect to the possible bleeding complications in the anticoagulated patients; in both, lobular hepatitis with bridging necrosis was described. Clinically/serologically, there was no evidence for autoimmune hepatitis, although it could not be excluded with certainty, as the autoimmune hepatitis score [14] could not be applied due to the lack of histology. However, relevant autoantibodies (antibodies to nuclei, actin, liver−kidney microsomes, soluble liver/liver−pancreas antigen, mitochondria, neutrophils) could not be detected, and immunoglobulin G (IgG) was normal. The relationship between liver disease and drug intake was further emphasized in these patients by the fact that liver enzymes and clinical symptoms normalized after withdrawal of the drug. Twenty-four patients received the drug for the first time, and six were accidentally reexposed. In one of them at first exposure, an increase of transaminases was noted. The time from the beginning of drug intake to development or registration of liver injury was significantly shorter in the reexposed patients [mean ± standard deviation (SD) 91± 81 days, median 90 days; range 3–180 days] than in the 24 patients who had received the drug for the first time (mean ± SD 500±845, median:150 days; range 1–3,700 days; $p<$ 0.05) Clinical, biochemical, and serological parameters of all 30 patients are shown in Table 1.

Furthermore, peripheral blood was obtained from 10 individuals, who were treated with phenprocoumon for at least 6 months but had no evidence for side effects toward this substance, as well as from 10 healthy individuals who have never received this drug.

Methods

Lymphocyte transformation test

From each patient, 30–50 ml heparinized blood was drawn for the LTT for diagnostic reasons. Peripheral blood mononuclear cells were isolated within 24 h by Ficoll-Hypaque centrifugation, as described [15, 16]. Cells were washed two times in Hank's solution and resuspended in 3 million cells/ml in Roswell Park Memorial Institute (RPMI)

Table 1 Clinical, biochemical and serological parameters in 30 patients in whom phenprocoumon-induced liver disease was suspected[a]

Parameters			
Age (years)	Mean ± standard deviation (SD)	65±13	
	Range	37–84	
Sex	Females:males	18:12	
Time of phenprocoumon intake (months): mean ± SD (median)	7±8 (6)[b]		
Biochemical parameters	Normal values	Mean ± SD (median)	Number (%) positive
ALAT (μkat/L)	< 0.58	6.52±6.25 (4.58)	26
ASAT (μkat/L)	< 0.58	4.78±4.55 (2.62)	27
AP (μkat/L)	< 2.8	5.67±6.21 (3.23)	16
γGT (μkat/L)	< 0.83	340±369 (204)	26
Bilirubin (μmol/L)	< 26	68.4±85.5 (34.2)	14
IgG (g/L)	< 18.00	11.41±3.93 (10.15)	1
IgA (g/L)	< 4.00	3.03±1.79 (2.52)	9
IgM (g/L)	< 2.80	1.52±0.97 (1.19)	3
Enzyme profiles (number patients)			
Hepatitic	15		
Cholestatic	12		
Mixed type	3		

ALAT alanine aminotransferase, *ASAT* aspartate aminotransferase, *AP* alkaline phosphatase, *γGT* gamma-glutamyl transpeptidase, *IgG* immunoglobulin G, *IgA* immunoglobulin A, *IgM* immunoglobulin M

[a] Autoantibodies indicative for an autoimmune liver disease (antibodies to nuclei, actin, soluble liver/liver−pancreas antigen, liver−kidney microsomes, mitochondria) could not be detected in any of the patients

[b] Excluding four patients who had taken the drug for more than 5 years

1640 culture medium supplemented with gentamicin and 25% autologous serum. For the proliferation assay, peripheral blood mononuclear cells (PBMC) (3×10^5/well) were seeded into 96-well cell culture plates and cultured without antigen, with pokeweed mitogen as a positive control, and with phenprocoumon in five concentrations ranging from 0.1 to 1.000 μg/ml for7 days at 37°C, 5% CO_2 in a humidified atmosphere. Over the final 18 h, the cultures were pulsed with ^{3}H-thymidine (0.74 Mbq/ml, 20 μl/well) and harvested onto fiberglass filters. The incorporated radioactivity was measured by liquid scintillation spectroscopy using a β-counter and given as counts per minute (cpm). All cultures were performed four- or five-fold; the mean cpm was used to calculate the stimulation index (SI): SI=mean cpm with antigen/mean cpm without antigen.

Laboratory parameters

Biochemical parameters were analyzed by standard methods. Quantitative immunoglobulins were determined using nephelometry (Beckmann Instruments, Munich, Germany).

Statistical analysis

For comparison of clinical data in different groups of patients, SPSS version 15.0 was used applying the nonparametric Mann−Whitney test. Differences with $p<0.05$ were considered statistically significant.

Results

Demonstration of sensitized lymphocytes in patients with phenprocoumon-induced liver disease

Fifteen of the 30 patients with suspected phenprocoumon-induced liver disease showed a positive result in the LTT (SI>2.0; range 2.1–17), that is, they had sensitized lymphocytes against this substance. The reaction was strictly dose dependent, showing positive results in most instances at 10 μg phenprocoumon/ml (Fig. 1); thus, in ten patients, the highest SI was observed with 10 μg, in four patients with 1,000 μg, and in one patient with 1 μg phenprocoumon/ml. Proliferation of lymphocytes at 10 μg/ml in the 15 LTT-positive patients was, therefore, significantly higher than the spontaneous proliferation or proliferation with phenprocoumon at higher or lower concentrations (Fig. 1).

Six patients had been reexposed to the drug, and four of them (67%) were positive in the LTT. Of the 24 patients who had received phenprocoumon for the first time, 11 (46%) revealed a positive LTT. Stimulation indices in the

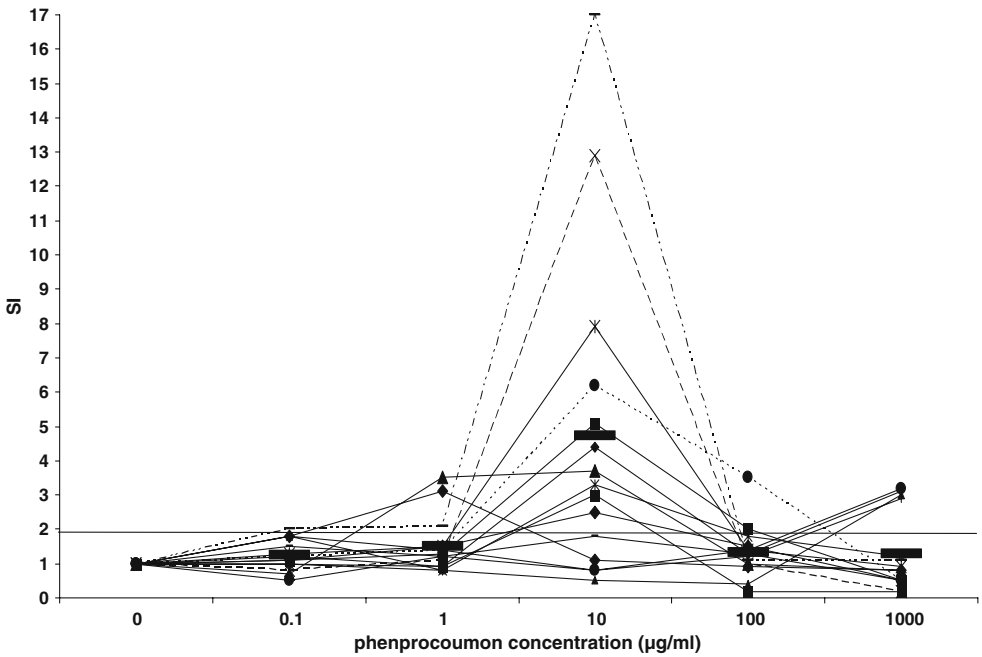

Fig. 1 Dose-dependent in vitro reactivity of lymphocytes from 15 patients with phenprocoumon-induced liver injury being positive with phenprocoumon in the lymphocyte transformation test ▬ = mean values

reexposed patients did not differ significantly from that in patients who had received the drug for the first time (mean ± SD: 2.82±21.38, median 2.5; vs. mean ± SD: 3.15±4.11, median 1.1; p=0.75). Nine patients with a positive LTT toward phenprocoumon took further drugs, which were also analyzed by LTT, but in none of the patients sensitized lymphocytes against these drugs were observed (data not shown). None of the ten patients who were treated with phenprocoumon but had no clinically evident side effects had sensitized lymphocytes against phenprocoumon, and the ten healthy blood donors were also negative in the LTT.

Comparison of clinical/biochemical parameters in LTT-negative and LTT-positive patients

There was no difference in the sex ratio in positive or negative patients in the LTT, but positive patients were significantly younger than the negative group (Table 2). Only one patient belonging to the group of LTT-positive patients had signs of hypersensitivity reaction (eosinophilia, fever, exanthema, vasculitis) in addition to liver injury. Furthermore, LTT-positive or -negative patients did not differ with respect to levels of liver enzymes or quantitative immunoglobulins. Of the 15 LTT-positive patients, seven had hepatitic, five cholestatic, and three a mixed enzyme profile; a similar distribution was observed in LTT-negative patients.

Moreover, lymphocyte reactivity (SI) did not correlate with duration of phenprocoumon therapy or time interval since withdrawal of the drug (for both correlation coefficient r<0.15).

Discussion

In this study, we could show for the first time in a larger group of 30 patients that in phenprocoumon-induced hepatitis, immunological mechanisms may play an important role. Thus, in 15 of 30 patients (50%) with hepatitis or cholestatic liver disease in whom phenprocoumon was highly suspected to be the causative agent due to appropriate time interval and history [13], sensitized lymphocytes against this substance were observed in vitro by LTT, revealing stimulation indices up to 17. These data confirm previous case reports showing either a positive LTT or activated lymphocytes by flow cytometry [5–7].

The LTT is nowadays accepted as the most consistent in vitro test for identifying a drug suspected of causing allergic disorders, especially hepatitis [5, 6, 10, 11, 17–19]. It is very specific [20] and correlates with the clinical manifestations. Thus, in our study, patients exposed to phenprocoumon but without signs of liver disease or other side effects, as well as healthy blood donors, were negative. This observation and the fact that the in vitro reaction of PBMC from patients with clinically manifest hepatitis was strictly dose dependent emphasizes the specificity of the test and excludes that the observed positive reactions are merely epiphenomena [10]. PBMC of two patients who were positive in the LTT with phenprocoumon could be also tested against two other coumarin derivatives (warfarin and acenocoumarol; data not shown). One patient showed a strong reaction to both substances, whereas the other was negative, indicating that different side chains of the molecules may be involved in the induction of immunological reactions. This may explain why reports about the

Table 2 Comparison of clinical, biochemical and serological findings in patients with suspected phenprocoumon-induced liver disease being either positive or negative in the lymphocyte transformation test (LTT)

Parameters		Patients		*P* value
		LTT negative (SI <2) (*n*=15)	LTT positive (SI >2) (*n*=15)	
Age (years)	Mean ± standard deviation (SD) (median)	71±11 (75)	60±13 (63)	0.023
	Range	51–84	37–83	
Sex	Females:males	9:6	9:6	
Time of phenprocoumon intake (months): mean ± SD (median)[a]		9±10 (7)	5±3 (6)	n.s.
Days from withdrawal of the drug until LTT[a]				
	Mean ± SD (median)	20±12 (15)	27±13 (24)	n.s.
	Range	9–48	8–46	
	Not withdrawn	5	2	
Number patients with reexposure		2	4	
Biochemical parameters	Normal values	Mean ± SD (median)	Mean ± SD (median)	
ALAT (μkat/L)	< 0.58	5.25±5.02 (4.20)	7.7±7.2 (6.65)	n.s.
ASAT (μkat/L)	< 0.58	4.43±4.23 (2.05)	5.12±4.97 (3.83)	n.s.
AP (μkat/L)	< 2.8	4.33±4.22 (3.40)	6.80±7.48 (2.31)	n.s.
γGT (μkat/L)	< 0.83	4.33±4.88 (3.30)	6.90±7.10 (4.85)	n.s.
Bilirubin (μmol/L)	< 26	85.5±119.7 (34.2)	34.2±34.2 (34.2)	0.098
IgG (g/L)	< 18.00	11.38±3.63 (10.40)	11.22±4.10 (9.42)	n.s.
IgA (g/L)	< 4.00	3.35±1.60 (2.90)	2.70±1.93 (1.64)	n.s.
IgM (g/L)	< 2.80	1.48±1.09 (1.19)	1.43±0.86 (1.10)	n.s.
Enzyme profiles	Number (%)			
Hepatitic		8 (53)	7 (47)	
Cholestatic		7 (47)	5 (33)	
Mixed		0	3 (20)	

ALAT alanine aminotransferase, *ASAT* aspartate aminotransferase, *AP* alkaline phosphatase, *γGT* gamma-glutamyl transpeptidase, *IgG* immunoglobulin G, *IgA* immunoglobulin A, *IgM* immunoglobulin M, *SI* stimulation index, *n.s.* not significant

[a] Excluding the four patients who had been treated with phenprocoumon for more than 5 years

safety of acenocoumarol in patients with phenprocoumon-induced liver diseases are contradictory [21–23].

The sensitivity of the LTT ranges between 15% and 80% and depends upon the group of drugs, their metabolites, and antigen presentation [20, 24]. With phenprocoumon, we achieved a sensitivity of 50%, that is, 15 patients with liver injury assumed to be due to phenprocoumon intake were negative in the LTT. There may be several reasons for this. First, false negative LTT results are quite frequent when the test is not performed within the optimal time interval after appearance of symptoms and withdrawal of the drug (14 days to 6 weeks) [10, 11]; however, in our study, we included only those patients in whom this precondition was fulfilled. Second, toxic and nonimmunologic processes may have been responsible for the side effects [25, 26]. Third, the allergic reaction is no induced by the drug phenprocoumon itself but rather by its metabolites (as, for instance, shown for metamizole-induced adverse reactions [15]), which could not be analyzed in our test system. Fourth, phenprocoumon was not the causative agent. Fifth, the LTT in its present form is not sensitive enough.

Several research groups have tried to improve LTT sensitivity by various modifications such as measuring cytokines in the lymphocyte supernatants, analyzing lymphocyte activation markers such as CD69 by flow cytometry or coculturing lymphocytes with cytokines such as alpha interferon [27–30]. We could not analyze these alternative methods systematically in parallel to the LTT. However, it is our experience with other drugs that LTT results do not correlate with cytokine production or expression of activity markers on lymphocytes (unpublished observations).

The identification of an allergic idiosyncratic drug reaction is currently a circumstantial diagnosis. Characteristics of type 1 allergic disorders such as fever, rash, or eosinophilia have not been described for coumarin-induced hepatopathy, which is rather a type IV (delayed-type hypersensitivity) reaction, and were present in only one of

our patients. Laboratory parameters are also no reliable indicators for phenprocoumon-induced liver injury, as it may manifest itself either with a hepatitic or cholestatic or even a mixed enzyme profile, as shown in this study. The LTT might therefore be a helpful tool to verify the diagnosis of phenprocoumon-induced liver injury, at least in 50% of patients, although one must be aware that this test can be performed only in specialized centers.

It remains unclear as to how immunological reactions in phenprocoumon-induced hepatitis are induced. A change in the antigenicity of hepatocytes caused by antigen expression of coumarin metabolites must be considered [3, 8, 9, 31]. Phenprocoumon is highly protein bound and hydroxylated and conjugated in the liver [32–36]. Cytochrome P450 (CYP) 2C9 and CYP3A are the major enzymes involved in the hepatic metabolism of phenprocoumon [32, 33, 37]. Genetic polymorphisms may alter hydroxylation of phenprocoumon, thereby influencing protein binding of its metabolites and formation of neoantigens. Side effects toward this drug may, therefore, depend upon genetic background, but this could not be analyzed in our study. However, environmental factors such as combination with other drugs, including herbal remedies; alcohol; obesity; or infectious processes may also facilitate a phenprocoumon-related allergic reaction [38–42].

The onset of phenprocoumon-induced liver disease apparently can occur at any time during treatment, according to the literature, between a minimum of 3 and a maximum of 10 [1] months. Latency is reduced to 7–50 days in the case of reexposure to the respective drugs. These data could be largely confirmed in our patients. However, it became evident that even after treatment of more than 5 years, (allergic) phenprocoumon-induced hepatitis can evolve. We also observed this phenomenon with other drugs, such as nonsteroidal antirheumatics or antiepileptics, used in chronic disorders (unpublished observation). Whether those late manifestations are facilitated by changes in exogenous factors (combination with other drugs; infections) has yet to be evaluated. Interestingly, in one patient who received phenprocoumon for the first time and had never been treated with other coumarin derivatives, DILD occurred as early as 24 h after intake of phenprocoumon. Considering the fact that coumarins also occur also in plants (e.g., woodruff) or tobacco [43, 44], one could surmise that contact with such environmental antigens led to presensitization and cross-reactivity with phenprocoumon.

In conclusion, we have shown that in a subgroup of patients with phenprocoumon-induced liver injury, allergic mechanisms may play an important role in the pathogenesis of the disease, as shown by a positive LTT. This test may, therefore, help to identify those immunologically mediated side effects. It still has to be evaluated in further studies whether it may also be useful to help exclude in vitro cross-reactivity with other coumarin derivatives such as warfarin or acenocoumarol.

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