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Pathophysiological characterization of drug hypersensitivity to tribenoside

Research Article

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Abstract: Background. Tribenoside is a semisynthetic sugar derivative that is mainly indicated for treatment of chronic venous insufficiency. Up to 10% of patients treated by tribenoside can suffer from skin side effects. The adverse effects usually present as angioedema, urticaria, or maculopapular exanthema. The pathophysiology of the reaction has not as yet been elucidated. Methods. In this study, we examined 22 patients with drug eruptions caused by tribenoside. Patch tests were performed to investigate in vivo cellular reactions. Laboratory investigations were carried out by lymphocyte transformation tests and basophil activation tests. Results. We found a positive patch test reaction to tribenoside in one patient. The lymphocyte transformation test elicited a borderline positive reaction in one patient, and the basophil activation test gave a clearly positive reaction in another patient. Conclusion. The diagnosis of drug hypersensitivity reactions is a challenge. Both delayed and immediate immunologic response may play a role in the etiology of tribenoside-induced exanthemas. Our investigation and results indicate that benzoic acid could be the antigenic determinant in drug hypersensitivity to tribenoside.

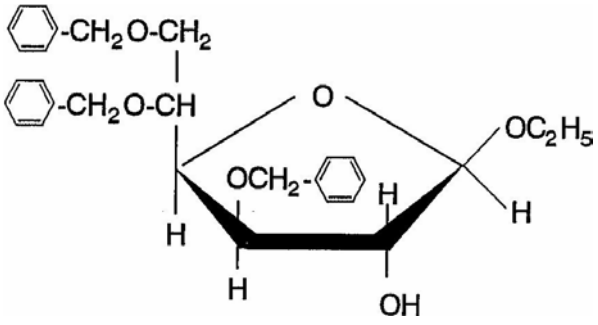
Keywords: *Basophil activation test • Benzoic acid • Drug hypersensitivity • Lymphocyte transformation test • Patch test • Tribenoside*

1. Introduction

Tribenoside is the semisynthetic sugar derivate ethyl-3, 5, 6-tri-O-benzyl-D-glucofuranoside, a glucose with one ethyl and three benzyl groups (Fig. 1). It is widely used in capsule (cps) form for oral administration and cream or suppositories for topical application under several brand names. Tribenoside is indicated for the treatment of chronic venous insufficiency, hemorrhoids, and arthritis [1]. It has a high affinity for the vessel wall and a wide spectrum of pharmacological effects, including venotonic, anti-edematous, fibrinolysis-promoting, analgesic, anti-inflammatory (inhibiting histamine and prostaglandins), anti-allergic, membrane-stabilizing, and bactericidal effects [2]. It regulates expression and localization of laminins in epidermal cells to reconstruct basement membranes in wound healing [3].

Tribenoside may induce adverse reactions as gastrointestinal symptoms and hypersensitivity reactions [4].

Figure 1. Chemical structure of tribenoside [4]



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Cutaneous adverse drug reactions (CADR) after oral use of tribenoside appear in up to 10% of treated patients [5,6]. Reactions to oral tribenoside occur as angioedema, urticaria, or various types of eruptions (macular, papular, multiform, or purpuric; less commonly, vesicular or nummular), typically of deep purple color. The onset of the rash is often from the third to the eleventh day of the drug exposure [7]. Tribenoside can also cause allergic contact dermatitis after topical application in the form of ointment. It manifests as edema, erythema or blisters, or itching in the treated area [8]. That the incidence of CADR caused by tribenoside is high, the pathogenesis has not yet been explained.

CADR can arise either from immunological or non-immunological mechanisms, though the preponderance of evidence suggests an important role for immunological responses. Most drugs are small molecules with a low molecular weight, as well is tribenoside at 478,6 Da. Therefore, these drugs are not recognizable by the immune system and are unable to induce an immune response in their native state. The molecule of drug must become a hapten by covalently binding to a protein, thereby forming a hapten-carrier complex, which then undergoes processing and presentation. Very few drug molecules are chemically reactive in nature without metabolism or binding to a protein (e. g. peicillins or cephalosporins). Most are chemically inert and must be metabolized or degraded to a chemically reactive form to undergo haptenation with cellular proteins (representing a prohapten). Recently, Pichler et al. provided an alternative mechanism for T cell activation, known as the p-i concept (**p**harmacological interaction of drug with **i**mmune receptors) [9]. According to this mechanism, T cells are directly stimulated by chemically inert drugs. The interface of the drug with T cell receptor is non-covalent, reversible, labile, stabilized by the major histocompatibility complex/peptide interaction, and independent on metabolism of the parent drug and antigen processing.

The immune response can involve every type of immune effector mechanism. The four main types of immune reactions can be classified, according to Coombs and Gell, into an immediate-type hypersensitivity reaction (mediated by drug-specific IgE antibodies bound on the surface of mast cells and basophils) generating urticaria, angioedema to systemic anaphylaxis, cytotoxic reaction and immune complex reaction resulting in vasculitis and urticaria (both mediated by drug-specific IgG or IgM antibodies), and delayed-type hypersensitivity reactions (mediated by drug-specific T cells) with very different clinical manifestations as maculopapular eruptions, erythema multiforme, toxic epidermal necrolysis.

We investigated the condition of our CADR patientsafter oral administration of tribenoside by available tests (a lymphocyte transformation test for the evaluation of specific cellular immunity such as type IV hypersensitivity reactions, delayed reactions), a basophil activation test for the detection of IgE-mediated hypersensitivity reactions (type I hypersensitivity reactions, immediate reactions) to demonstrate either a delayed or immediate hypersensitivity reaction role in the development of the skin eruption.

2. Patients and methods

2.1. Patients

The cohort consisted of 22 patients (15 women, aged 25–77 years; and 7 men, aged 58–84 years) with CADR attributed to oral tribenoside (Glyvenol cps. 400 mg; Novartis, Prague, Czech Republic) (Table 1). The diagnosis was based on a detailed history, clinical features, and course of CADR. We divided patients' CADR according to the type of rash and course of exanthema into apparent immediate and delayed reactions. The onset of the eruptions typically occurred after 7 to 12 days (range 6–30 days) and were classified as immediate reactions, whereas generalized macular, papular or multiform exanthema with a dark red or purple colour, either persisting for a long time or healing within 1–2 weeks after discontinuation of the drug, were considered as consistent with the delayed type of hypersensitivity reaction. Maculourticarial exanthema of short duration was considered to be an immediate hypersensitivity reaction.

The rash was macular or papular in 14 patients (Table 1, Fig. 2a, 2b), multiform in 4 patients, and maculourticarial in 4 patients. Other symptoms accompanying exanthema included purpura of the lower legs (1 patient), angioedema of the palms, feet and auricles (1 patient) and face (1 patient), enanthema in the oral cavity (1 patient), fever (1 patient), and swollen throat (2 patients).

All the patients tested signed their informed consent according to Helsinki declaration.

2.2. Methods

Patch tests (PTs) were performed according to the guidelines for skin testing in CADR [7,10,11]. Reactions were evaluated at 20 minutes to avoid immediate reactions, on day 2 (48 hours), day 4 (96 hours), and if negative, on day 7. Reactions were scored as recommended by the International Contact Dermatitis Research Group [12].

Table 1. Characterization of patients. Pt. – patient, N – negative, P – positive, M – male, F – female, IR – immediate reaction

Pt. No.	Sex	Age (years)	Clinical features, exanthema	Onset of CADR (days/years)	Other new drug (intake in days)	PT	LTT	BAT
1	F	45	maculopapular	13	0	N	N	N
2	F	59	papular	9	0	N	N	N
3	M	60	maculopapular	8	0	N	N	N
4	M	84	maculopapular	30	tiapride (40)	N	P	N
5	F	37	multiform	10	0	N	N	N
6	F	50	maculourticarial	14	0	N, IR	N	P
7	F	61	multiform, oedema of face, throat tightness	7	0	N	N	N
8	F	54	maculopapular	6	0	P	N	N
9	M	74	papular	8	0	N		
10	F	74	maculopapular	12	0	N		
11	F	77	macular	12	0	N		
12	F	37	multiform, enanthema, throat tightness	10	0	N		
13	F	50	papular	10	0	N		
14	M	58	maculourticarial	yrs	0	N		
15	F	63	multiform, fever	yrs	0	N		
16	M	70	papular	yrs	0	N		
17	F	68	maculopapular	yrs	0	N		
18	F	57	maculourticarial	yrs	0	N		
19	M	72	papular, purpura	18	0			
20	F	25	maculourticarial, edema of palms, feet, auricles	30	0			
21	F	26	papular	8	0			
22	M	68	papular	yrs	0			

PTs were performed with the commercial form of the drug (Glyvenol cps., tribenoside dissolved in 85% glycerol) diluted in white petrolatum and alcohol at 30% concentration according to the guidelines for drug patch testing [7]. Curatest tapes were used (Lohmann & Rauscher, Rengsdorf, Germany). White petrolatum was applied as the negative control; parabens (Paraben mix Mx-03C, Chemotechnique Diagnostics, Vellinge, Sweden) were tested as conservatives. The cover of the gel capsule was tested to exclude any reaction to one of its components (ethanol, glycerol, parabens, gelatine, canthaxanthin). Exposure to the allergens lasted for 48 hours.

The lymphocyte transformation test (LTT) measures the proliferation of T cells in peripheral blood sensitized by a given antigen [13]. The LTT was performed in heparinized blood (20 ml) from which peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation through Ficoll density gradient (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). PBMCs were resuspended in culture medium ($1x10^6$ cells/ml, RPMI-1 640 medium [Sigma Aldrich, Steinheim, Germany] supplemented with L-glutamine, penicillin, streptomycin (Sigma Aldrich, Steinheim, Germany), and 10% heat-inactive autologous serum). Cells were incubated with the antigen (tribenoside obtained from Glyvenol cps. dissolved in 96% alcohol) in different concentrations (50, 10, 1, 0.1 µg/ml; determined by the results of a toxicity test), with pokeweed and phytohaemagglutinin mitogen (PWM and PHA, respectively; Sigma Aldrich, Steinheim, Germany, 5 µg/ml, at an optimal time period of 6 and 3 days) as positive control for lymphocyte activation, and cells alone in culture medium as negative control for 6 days under standard conditions (at 37^0 C in

Figure 2a. Generalized papular CADR – back of the patient No. 21

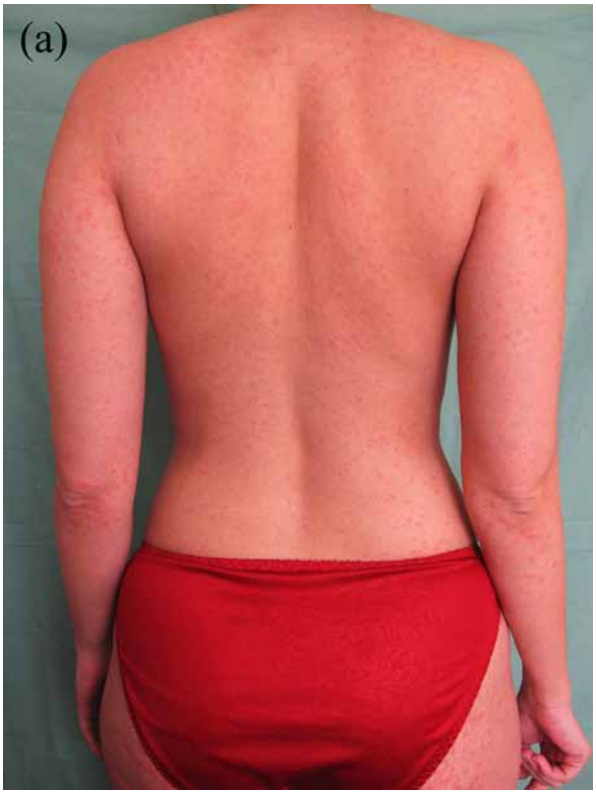
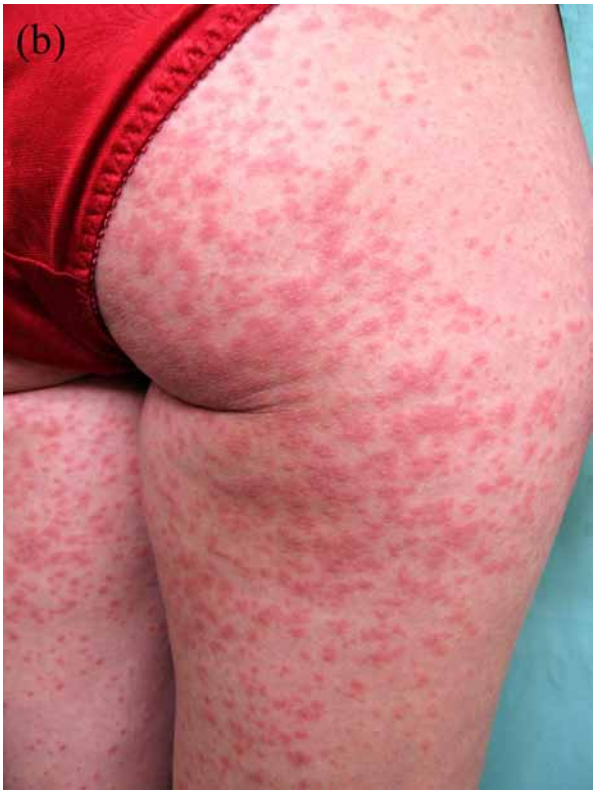


Figure 2b. Generalized papular CADR – thigh of the patient No. 21



a 5% CO_2 incubator). These values of the drug concentrations were evaluated in a toxicity test that determines a non-toxic concentration of the tested antigen. Different concentrations of the drug (1,000 to 1 µg/ml, according to the experience of Pichler et al. [14]) were added to the PBMC of 4 non-allergic donors. Cells were stimulated with mitogen phytohaemagglutinin (PHA, 5 µg/ml). Only drug concentrations that did not inhibit the PHA-induced proliferation by more than 15% could be used (Fig. 4). As a control, the PBMC were cultured alone in medium (negative control that shows only spontaneous proliferation) and with PHA (positive control, polyclonal non-specific mitogen-stimulated T lymphocytes with optimal time period of 3 days).

All examinations were performed in triplicate; radiolabeled ^{3}H-thymidine (Nuclear Research Institute, Řež, Czech Republic) was added 16 hours before the end of the cultured period (6 days). ^{3}H-thymidine was incorporated into the newly formed DNA of dividing T cells. The radioactivity was measured in a beta scintillation counter (Tri-carb 2 100 TR, liquid scintillation analyser, Canberra Packard, Meriden, CT, USA). The result was expressed as a stimulation index (SI) which was calculated as the relationship between mean cpm (counts per minute) in antigen-stimulated cells compared with mean cpm in control cells without antigen. The test was considered as positive if the SI had a value greater than 2 [14,15,16].

The basophil activation test is an in vitro functional test based on the quantification of basophil activation by flow cytometry (Cytomix FC500, Beckman Coulter, Miami, FL, USA). Specific activated markers (CD63, CD203c) were detected by monoclonal antibodies (CD63-FITC, CD203c-PE, Immunotech, Beckman Coulter Company, Marseille, France).

Briefly, whole heparinized blood (500 µl) was incubated at 37^0 C with allergens for 15 minutes. Tribenoside diluted in RPMI 1 640 medium in a concentration of 50 -10 -1 µg/ml was used as an allergen. IL-3 (recombinant human IL-3, BD Bioscience Pharmingen, San Diego, California, USA) was added pre-incubation to enhance the reactivity of basophils. Two controls were used: a negative control (cells alone in medium) and a positive control (cells with medium and FMLP [chemotactic peptide formyl-methionin-leucyl-phenylalanine, Sigma Aldrich, Steinheim, Germany]).

Results were expressed as the percentage of activated basophils among the total basophil number labeled with anti-IgE antibodies (IgE/PE-DY647; Exbio, Prague, Czech Republic) and CD203c after incubation with the allergen. Results were considered positive if at least 2 sequential concentrations of the allergen induced

more than a 10% increase of activated basophils above the control values (positive result with more than 15% of activated basophils; negative control below 5%; and positive control above the value of positive result) [17,18].

3. Results

PTs were performed in 18 of 22 patients (4 patients refused further examination).

Patient characteristics are shown in Table 1. Fifteen patients used tribenoside as a new drug continually (range 6 to 30 days); one patient used tribenoside as a new drug while taking another new drug (tiapride). In these two groups, the interval between the start of tribenoside and the onset of the CADR was 7 to 12 days in most cases, ranging from 6 days to one month. In 6 patients the exanthema occurred after a long-term use of tribenoside (up to several years), other causes were excluded. A history of allergy was positive in 14 of 22 patients, most frequently to aeroallergens, wasp, bee or food allergy (nuts, pineapple) and drug allergy (penicillin, tetracycline, iodine, neomycin and procaine).

A positive patch test reaction to tribenoside was seen in patient 8 (Fig. 3), confirming delayed hypersensitivity. It corresponded with the onset of the eruption after 6 days and clinical features of generalized maculopapular exanthema, healing within 1 week. This patient had history of allergy to aeroallergens and penicillin.

Papular itching exanthema on the arm and forearm was elicited during after 10 hours in patient 6, the PT itself was negative. However, a maculourticarial type of exanthema and a positive result of BAT indicated immediate reaction in pathogenesis of CADR in that patient.

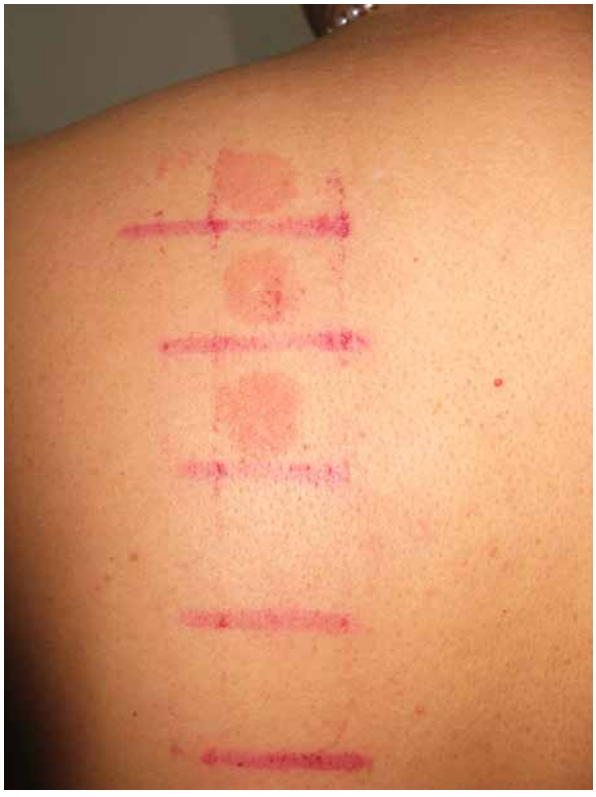
Tests with parabens and petrolatum were negative in all patients. Results of skin testing with cover of capsule were negative in 17 of 18 patients. The only one positive reaction was seen in patient 8.

A basic laboratory check (blood count, ESR, liver and kidney function test, blood glucose, IgE, urinalysis) was performed in all patients. Elevated IgE was found in 7 patients (26%) (104 – 6,637 IU/l) (normal limit 0–100 IU/l). The other lab results were within normal range.

The lymphocyte transformation test and the basophil activation test were performed in 8 of 22 patients (all with PTs).

In LTT, the appropriate drug concentration was first tested a toxicity. Tribenoside at a concentration of 1,000 µg/ml proved to be lymphocytotoxic (Fig. 4). It inhibited PHA-stimulated proliferation of cells compared with the positive control. The dpm value (disintegrations per minute; dpm = cpm/efficiency) of cells incubated with tribenoside at 1,000 µg/ml plus PHA was similar as the negative control. The dpm value cells incubated with tribenoside at 50 to 0.1 µg/ml plus PHA was similar the positive control these concentrations of tribenoside were used for the examination of the patients.

Figure 3. Positive result of PT with tribenoside. Glyvenol at 30% in alcohol: ++, Glyvenol at 30% in petrolatum: ++, Glyvenol cps.: ++, Petrolatum: -, Parabens: -



The toxicity test with 96% alcohol (used by dilution of tribenoside) and 85% glycerol (component of the capsule) was negative (data not shown). One borderline positive result of LTT was found in the patient 4. The SI value in tribenoside concentration 1 µg/ml was 2.45, and in 10 µg/ml was 1.82 (Fig. 5). The PT was negative. The results of LTT in the other patients were negative, including the patient 8, who had a positive PT.

The basophil activation test yielded a positive result in patient 6, in whom the papular exanthema on arms and forearms was elicited during (Table 2).

Table 2. Positive result of BAT in patient No. 6

Expression of marker	CD63 %	CD203c %
Negative control	3	10
Positive control	50	54
Glyvenol 10 µg/ml	30	42
Glyvenol 50 µg/ml	20	24

Figure 4. Toxicity test of Glyvenol. The relevant results of one of 4 non-allergic donors. The cell culture is measured in triplicates (mean ±SD) as described in Methods. PHA – phytohaemagglutinin, SD – standard deviation, dpm – disintegrations per minute.

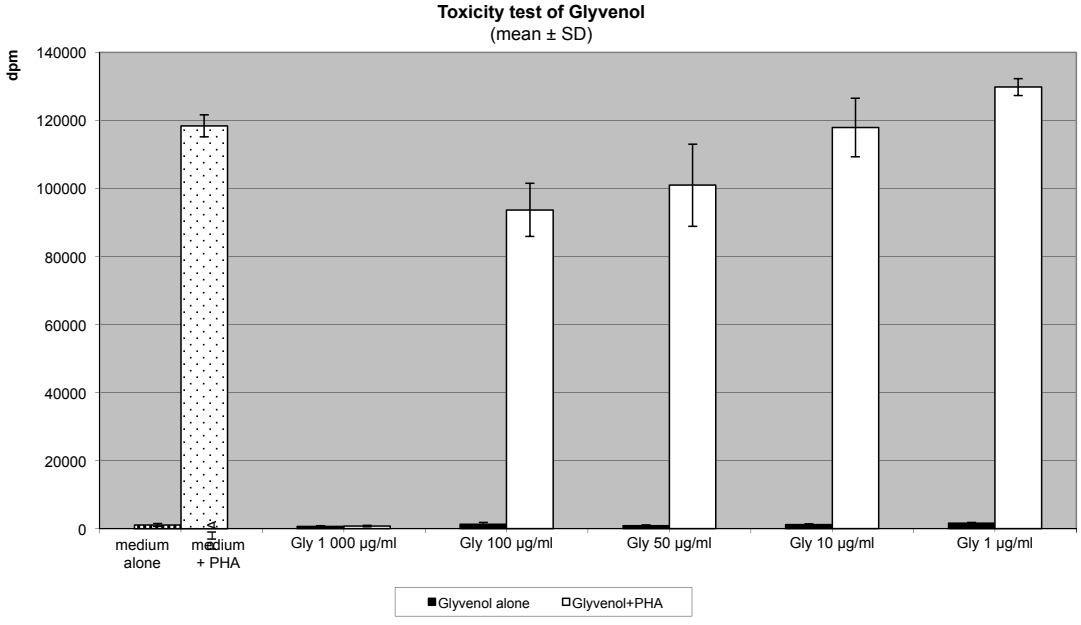
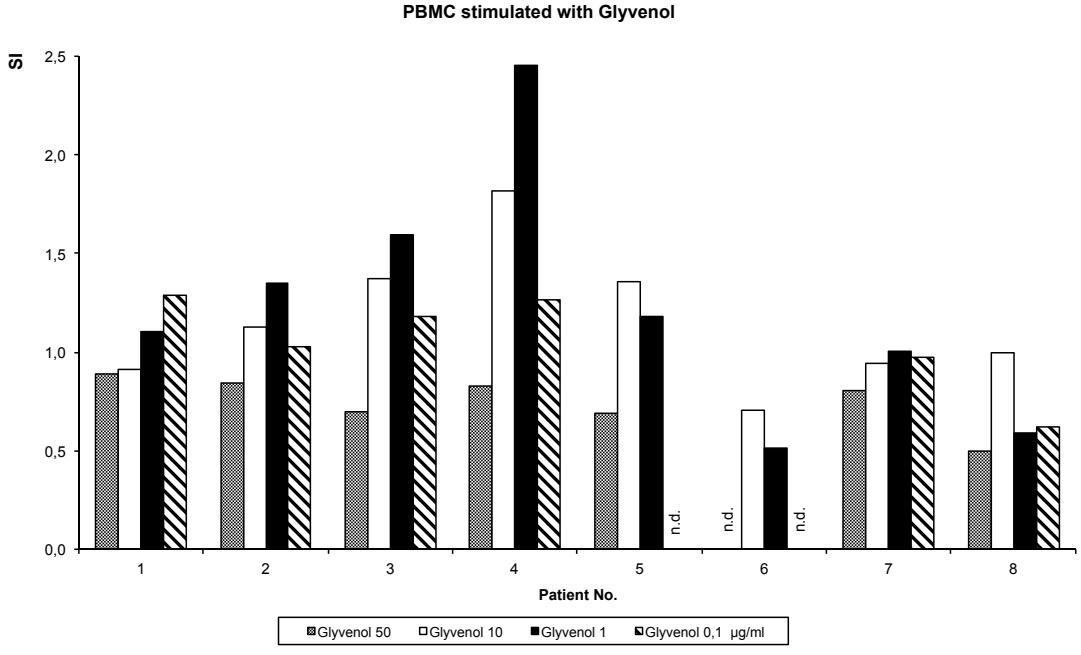


Figure 5. Results of LTT. Peripheral blood mononuclear cells (PBMC) stimulated with Glyvenol. The culture is measured in triplicates as described in Methods. SI means stimulation index..



4. Discussion

The pathogenesis of cutaneous adverse drug reaction induced by tribenoside administered orally has not yet been described. Therefore, we used a set of different *in vivo* and *in vitro* immunological tests with sensitivity also to the hapten and systematically examined the immunoreactivity of this drug.

Tribenoside is a sugar derivate that does not have antigenic properties such as those of protein antigens but may be able to act as a hapten or prohapten. Its prohapten must be metabolized to a chemically reactive form before it is able to bind covalently to a protein

carrier as a hapten and thereby induce an immune response. Tribenoside is metabolized very intensively by oxidation. Three benzyl groups in the C3, C5 and C6 positions of the glucose are oxidated to benzylalcohol, then to benzylaldehyde and to benzoic acid [19]. During this oxidative process, three molecules of benzoic acid are released from one molecule of tribenoside. Benzoic acid is detoxified as hippuric acid by conjunction with glycine in the liver. In the course of glycine conjugation, benzoic acid is successively converted into benzoyl-CoA and benzoylglycine by mitochondrial enzymes (benzoyl-CoA synthetase and benzoyl-CoA/glycine *N*-acyltransferase, respectively), utilizing ATP, CoA, and glycine [20]. The first step of oxidation is catalysed by alcohol dehydrogenase in the cytosol. In the second step, aldehyde dehydrogenase is involved. The other enzymes were described in the oxidation of aromatic aldehydes to their carboxylic acids by the P450 system in endoplasmic reticulum (in particular P450 of the 2B, 2C and 3A subfamilies and poorly in 1A and 2E) [21,22]. Tribenoside is eliminated by urine as hippuric acid (in 20%) and its metabolites formed by the oxidation (in 80%) [19].

The molecular structure of tribenoside, its metabolism and immunological results of antigen-specific BAT and LTT in sensitized patients, in which only slightly positive increased SI about 2 (in pacient No. 4) were found in comparison with the high SI in conventional protein antigens [15] indicate the reactivity of hapten and suggest that the reactive antigenic determinant is benzoic acid. These findings are fully in accord with previous investigations [2,8,19]. Moreover, benzoic acid as the antigenic determinant in drug hypersensitivity to tribenoside may explain the relatively high occurrence of observed reactions in patients treated with tribenoside. The patient may have been sensitized even before the drug was first used therapeutically, because benzyl alcohol and benzoic acid are naturally occurring aromatic constituents of balsam of Peru (myroxylon pereirae) and other botanicals [23]. The cross-reactivity of benzoic acid with myroxylon pereirae has been described [24]. Further, these aromatic compounds are used as additives in certain foods (E 210, also as salt of benzoic acid, sodium benzoate–E 211) and occur as a preservative in some drugs. They are widely used in cosmetics and also in the local treatment of burns, wounds and haemorrhoids. They have been clinically associated with hypersensitivity in allergic contact dermatitis, systemic contact dermatitis, contact urticaria and phototoxic reaction [25]. Immediate reaction has also been reported [26]. All this is in agreement with our findings and with BAT results. Prevalence of myroxylon pereirae sensitization ranges from 3.2% to 11.8% in the population [25]. The cross-reactivity is reported also with propolis, another substance widely used in “natural” cosmetics [27].

The diagnosis of drug eruptions is difficult and complex because of the limited possibilities for verification by clinical or laboratory tests. Use of laboratory methods (TPL and BAT) in the case of drug hypersensitivity diagnosis is still controversial in routine practice, as with all other methods now available for its low yield and high costs. A careful deliberation should be preceded by examination of the patient. It is essential to take into account clinical features of CADR and hence pathogenesis. In the second step, we consider properties of the tested drug and the factors that affect its use in the test (especially drug metabolism). PT has to make sense only in the CADR, which were mediated by T cells and occurred on the skin.

PTs and LTT do not overlap but they are complementary [14], as we saw in patients 4 and 8 who had delayed type hypersensitivity, first determined by history and clinical features as generalized maculopapular exanthema and then confirmed by PT or LTT. The combination of both methods increases sensitivity to 76%. LTT has higher sensitivity compared with a PT, 60% to 70%, but this value results mainly from values established for penicillins; the sensitivity of the PT is 20 to 40%, but reported values range from 7.5% to 86.7%.

The specificity of LTT is about 85% [14,28-31]. Detection of drug-specific T cells in vitro differs from inducing a local T cells-mediated inflammatory reaction during . A strong immune reactivity is frequently associated with clinical symptoms [14]. The other debated aspect of LTT is the duration of sensitization and the right time to test. Most authors are in agreement that the optimal time period between development of symptoms and carrying out a LTT is 4 to 8 weeks. During the acute stage of hypersensitivity, T lymphocytes are hyperstimulated; after this time period, the lymphocytes migrate to lymph nodes and their number in the peripheral blood decreases. However, in some cases sensitization may be detected after many years (by PT or LTT) [14,32]. We can confirm these findings: we have performed the LTT after a longer period of time (5 to 20 months) after the CADR, depending on patient compliance. The lymphocyte reactivity of our patient 8 was confirmed by a repeated PT. The first PT with a positive result was performed 5 months after the CADR. PT reactivity remained after 13 months, at which time the PT was repeated with a positive result.

It is recommended to perform a PT after complete resolution of the eruption, 6 weeks to 6 months, and after discontinuation of immunosuppressive therapy (systemic corticosteroids, at least 1 month) [7]. We have

performed the PTs 6 weeks to 24 months after the CADR resolution. It has been recommended to take blood samples for BAT at least 2 weeks after a hypersensitivity reaction and at least 1 week after discontinuation of anti-allergic medication.

Results of PT in patients may be influenced by the vehicle, drug concentration, skin sites where the tests were performed, epidermal absorption of the drug, drug metabolism, and concomitant factors (e.g., viral infection at the time of CADR) [7]. Although the sensitivity of PTs in CADR is low, they may confirm the culprit drug when chronological criteria are insufficient.

The positive test to the gel capsule in one patient (the patient had a positive test to tribenoside) was probably due to the contamination of the capsule by remnants of tribenoside. The cover of the gel capsule contains ethanol, glycerol, parabens, gelatine and canthaxanthin (a reddish-orange dye with antioxidant activity belonging to carotenoids). Parabens were tested with negative results.

LTT showed borderline positivity in one case (patient 4). Positive results of LTT in drug allergy should exceed the SI value 2. There are several possibilities that might explain our results of LTT mainly the chemical structure of the drug and the drug metabolism in human body that are critical for antigenic stimulation. In theinstance that tribenoside stimulates the T cells by the prohapten mechanism, the recognition of the native antigen in LTT by T cells is decreased and the lymphoproliferative response cannot be induced. A low but clearly detectable increase in a very sensitive specific radioactive proliferative assay indicates hapten reactivity. The sensitivity of LTT can be even enhanced by addition of mouse liver microsomal enzymes [29,33] that can metabolize the drug.

Positive results of the BAT were expected as the immediate reaction to pathogenesis of maculourticarial CADR (one positive result was detected in the patient who developed an immediate reaction during). In this patient the negative result of the LTT can be explained by a different pathogenesis of CADR (immediate reaction of hypersensitivity). Nevertheless, some authors have shown positive results LTT in IgE immediate reactions and supposed interconnection between Th1 and Th2 immune system, and the participation of T cells in the immediate reaction. Th1 and Th2 reactions simultaneously [14].

The results of complex functional tests in our patients prove the possibility of developing delayed and immediate type of immunopathogenic response in the etiology of tribenoside-induced exanthemas. This finding is in agreement with the clinical picture of exanthemas, which are also not uniform.

5. Conclusion

CADRs are a severe problem with a high occurrence (5% of all skin diseases), increasing incidence, and limited diagnostic methods. The history of CADR, its clinical course and picture, are essential for the determination of the type of immunological reaction and the appropriate choice of diagnostic tests.

We consider allergological and immunological examinations together with PTs as valuable and necessary essential contributions to the diagnostics of CADR that can elucidate the pathogenesis of drug eruptions. We have used a complex array of tests for specific immunological examinations. Their results indicate that benzoic acid can be the antigenic determinant in drug hypersensitivity to tribenoside, with hapten reactivity as the possible pathogenetic reaction. These metabolites and related compounds could be used in diagnostic tests and in this way, the sensitivity of the diagnostic process could be increased. The methodology of preparation and application of antigen will be further elaborated for routine use and to better understand the immunological mechanisms leading to hypersensitivity to individual drugs. This should also contribute to our work.

Conflicts of interest

None declared

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