0.7%, and 108.6 ± 2.2% for theophylline, ephedrine hydrochloride, and phenobarbital, respectively.

The HPLC method was applied to the assay of commercial tablets (Table II). Again, the compendial results for ephedrine hydrochloride and phenobarbital were higher than those obtained by the proposed HPLC method. By coincidence, the theophylline content in the three commercial tablet samples investigated was low; the values were around the minimum limit of the compendial potency requirement range of 90.0–110.0% of the label claim. Figure 2 shows a typical chromatogram of the assay solution from commercial tablets.

The HPLC method is more accurate and precise and considerably less time consuming than the compendial method. The wide detector response range between theophylline and phenobarbital was due to the large difference in concentration and posed no problems. The change in the detector attenuation during the chromatographic run did not affect the results. The wide variation in polarity, which necessitated the prior separation of ephedrine in the GLC methods (4, 5), presented no problems under the proposed experimental conditions. The method also is applicable to the assay of individual tablets since the procedure is based on the quantities of drugs present in one tablet.

REFERENCES

(1) "The United States Pharmacopeia," 20th rev., United States Pharmacopeial Convention, Rockville, Md., 1980, p. 784.

(2) J. L. Lach, T.-F. Chin, and E. L. Parrott, *Am. J. Hosp. Pharm.*, **29**, 1043 (1972).

(3) M. Elefant, L. Chafetz, and J. M. Talmage, *J. Pharm. Sci.*, **56**, 1181 (1967).

(4) H. W. Schultz and C. Paveenbampen, *ibid.*, **62**, 1995 (1973).

(5) J. L. Cohen, in "Analytical Profiles of Drug Substances," vol. 4, K. Florey, Ed., Academic, New York, N.Y., 1975, p. 466.

(6) M. K. Chao, K. S. Albert, and S. A. Fusari, in *ibid.*, vol. 7, 1978, p. 359.

(7) F. Bailey and P. N. Brittain, *J. Chromatog.*, **83**, 431 (1973).

(8) V. D. Gupta and O. H. Shek, *Am. J. Hosp. Pharm.*, **33**, 1086 (1976).

ACKNOWLEDGMENTS

Presented at the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, St. Louis meeting, April 1981.

Immunological Studies of Poisonous Anacardiaceae: Production of Tolerance in Guinea Pigs Using 3-*n*-Pentadecylcatechol-"Modified" Autologous Blood Cells

EDNA S. WATSON ˣ, JAMES C. MURPHY, PHILIP W. WIRTH, MAHMOUD A. ElSOHLY, and PAUL SKIERKOWSKI

Received July 7, 1980, from the *Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, MS 38677.* Accepted for publication December 15, 1980.

Abstract □ The development of contact sensitivity to poison ivy urushiol in guinea pigs was prevented by intravenous injection of 3-*n*-pentadecylcatechol (I) coupled to autologous blood cells. Hartley, linebred, guinea pigs were treated with pentadecylcatechol-"modified" blood cells or sham-treated blood cells 2 weeks prior to attempted topical sensitization with I. Skin testing of all guinea pigs with 3-, 1-, and 0.3-μg doses of I applied in 5 μl of acetone to abdominal skin sites was begun 2 weeks after attempted sensitization and repeated at 2- or 4-week intervals thereafter for 6 months or until study termination. Profound tolerance to I was observed at all skin testing intervals in the group receiving haptenated red cells and did not weaken substantially with time. Contact sensitivity to I in control animals, however, waned with time; the study was terminated at 6 months because of the low sensitivity level of the control animals at that period. Complete or partial tolerance was induced in ~80% of the treated animals. The immune tolerance obtained by the single injection of pentadecylcatechol-associated red blood cells was of long duration and urushiol specific. This treatment also conferred tolerance to three unsaturated congeners of I. The allergenic potencies of the pentadecylcatechols declined with increasing saturation of the alkyl side chain. Binding studies using tritiated pentadecylcatechol showed that 81% of the activity incorporated into the red cell was membrane associated and that 19% was cell sap associated.

Keyphrases □ Urushiol—production of tolerance using 3-*n*-pentadecylcatechol with autologous blood cells, guinea pigs □ Poison ivy—production of tolerance to urushiol using 3-*n*-pentadecylcatechol with autologous blood cells, guinea pigs □ 3-*n*-Pentadecylcatechol—production of tolerance to urushiol, guinea pigs □ Immunology—production of tolerance to urushiol using 3-*n*-pentadecylcatechol, guinea pigs

Poison ivy (*Toxicodendron radicans*), poison oak (*T. diversilobum* and *T. quercifolium*), and poison sumac (*T. vernix*) are the main causes of contact dermatitis in the United States. One or more of these species is present in almost every state in the continental United States. These plants are so prevalent and insidious that ~80% of the U.S. population is allergic to them and 50% are clinically sensitive, *i.e.*, react to 2 μg of urushiol or less (1).

BACKGROUND

The dermatitogenic principles contained in the resin of these plants are a group of chemically related catechols, commonly referred to as urushiols, differing mainly in the length and degree of unsaturation of the 3-*n*-alk(en)yl side chain. Poison ivy urushiol was shown to be mainly (>95%) a mixture of 3-*n*-pentadec(en)ylcatechols with zero, one, two, or three double bonds in the C_{15} side chain (2–7). Poison oak urushiol, however, consists mainly (>98%) of the C_{17} homologs (2, 3, 6). A small percentage of the C_{15} congeners was found in poison oak urushiol, and a small percentage of the C_{17} homologs was found in poison ivy components (2, 3, 6). The analysis and identification of poison sumac urushiol are incomplete.

Contact of these catechols with the skin of susceptible individuals results in sensitization to all urushiols of the plant family Anacardiaceae (8–10). Once sensitivity is developed, it is difficult, if not impossible, to eliminate. Hyposensitization by administration of plant extracts is not regularly obtained. It requires large doses and months or years to be produced, and sensitivity is rapidly regained upon cessation of treatment (10, 11). The albino guinea pig is the animal of choice for studying sensitivity to these allergens (12). Tolerance to poison ivy was reported to be produced in guinea pigs by subcutaneous injection of pentadecylcatechol (I) in mineral oil or by oral administration of large doses of I prior to attempted sensitization (13). However, since urushiols and I itself are potent primary irritants and skin sensitizers, their use for producing tolerance in nonsensitive humans is heavily compromised.

Recently, a series of ring-substituted derivatives of I was studied for potential use in the production of immune tolerance to contact sensitivity in guinea pigs (14, 15). Some 6-substituted derivatives were reported to

0022-3549/81/0700-0785$01.00/0

Table I—Evaluation of Skin Test Responses Using Draize Scoring System [a]

Response	Value
No erythema	0
Erythema and eschar formation	
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injury in depth)	4
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (area raised ~1 mm)	3
Severe edema (raised ~1 mm and extending beyond area of exposure)	4

[a] Maximum summed erythema and edema scores = 8.

produce tolerance to I and to have low sensitizing and skin irritation potential (14).

Recent studies showed that sensitization to contact sensitizers by the cutaneous route requires presentation of the contact allergen to the immune system by Langerhans cells present in the skin (16). When initial exposure is oral or parenteral by routes other than topical, immune tolerance to the sensitizer usually develops (14, 17, 18).

The discovery that intravenous injection of some skin sensitizers or closely related chemicals would prevent subsequent sensitization to the sensitizer suggested that the resulting tolerance may be mediated by coupling of the hapten with some blood components (19). Subsequent studies showed that membrane-coupled antigen (haptenated spleen, red, lymph node, thymic, or peritoneal exudate cells) is the tolerogenic form (20, 21).

The present report describes the induction of tolerance to poison ivy urushiol by intravenous injection of pentadecylcatechol-associated autologous red blood cells 2 weeks prior to attempted contact sensitization with I. Tolerance to the mono-, di-, and triolefinic congeners of I also was produced by this treatment. Tolerance induced by pentadecylcatechol-associated red cells was specific for poison ivy urushiol and not due to a general lack of immunocompetence of the tolerized animals. Radioisotopic studies using tritiated pentadecylcatechol as tracer showed that the majority of cell-bound pentadecylcatechol (81%) was associated with the red cell membrane and that the remainder was associated with the soluble cell sap.

EXPERIMENTAL

Animals—Female, Camm-Hartley[1], line-bred guinea pigs, 450–500 g, were used. Guinea pig food[2] and water supplemented with ascorbic acid were provided *ad libitum*.

Preparation of Pentadecylcatechol—Poison ivy urushiol purified as in a previous study (3) was reduced by catalytic hydrogenation, and the resulting pentadecylcatechol was purified by passage over a dry silica gel 60 (2) column with chloroform as the eluting solvent. Fractions were collected and tested for I using 1% alcoholic ferric chloride. Fractions containing I were combined, and the solvent was evaporated. The resulting residue was chromatographed on a MN-polyamide SC 6[3] column (particle size <0.07 mm) with 90% ethanol–water as the eluting solvent. Fractions containing I were combined. The solvent was evaporated, and the residue was crystallized from hexane to give colorless needle crystals of I, mp 58–59°. The identity of I was determined by GLC and GC–mass spectrometry.

Preparation of Poison Ivy Urushiol Congeners—The mono-, di-, and triolefinic components of poison ivy urushiol were isolated from purified poison ivy extract by converting the urushiol to its acetate derivative with acetic anhydride and pyridine. Urushiol acetate was passed over a silica gel G column impregnated with 5% $AgNO_3$, using 1% methanol in chloroform as the solvent system. The polarity of the eluting solvent was increased gradually to 4% methanol in chloroform; fractions containing the mono-, di-, or triolefinic congener acetates were pooled based on their TLC similarities in silver nitrate-impregnated silica gel plates. Each olefinic acetate was converted to the corresponding allergenically active congener by hydrolysis with sodium carbonate in dioxane–water solution (4:1). The reaction mixture was neutralized, and the free catechols were ether extracted. The identity and purity of the olefinic congeners were determined by GLC and GC–mass spectral analysis of the trimethylsilyl derivatives (22).

Table II—Method for Calculation of Degree of Delayed-Type Hypersensitivity

	Erythema and Edema Scores					
	24 hr		48 hr		72 hr	
Animal	Erythema	Edema	Erythema	Edema	Erythema	Edema
Group A						
01	0	0	2	1	2	0
02	1	0	3	2	3	1
03	0	0	0	0	0	0
04	2	2	4	4	4	3
Average (number of responders = 3)	1.0	0.6	3	2.3	3	1.3
Erythema and edema sum	1.6		5.3		4.3	
Degree of delayed-type hypersensitivity = 4.4						

Tritiated Pentadecylcatechol—Fifty milligrams of I containing 50 μg of the triolefinic congener, 3-*n*-pentadeca-8,11,14-trienylcatechol, was tritium reduced[4]. The specific activity was 10.6 mCi/mg.

Pentadecylcatechol Solutions—Solutions used for red cell modification consisted of 2 mg of I/ml of propylene glycol. Sensitizing solutions of I were prepared in acetone at a concentration of 1 mg/0.15 ml. All skin test solutions were prepared in acetone to contain 3, 1, or 0.3 μg of I/5 μl, except for the olefinic congeners which were prepared in acetone at 3 μg/5 μl. All solutions were analyzed for purity prior to use.

Preparation and Injection of Pentadecylcatechol-"Modified" and Sham-Treated Red Blood Cells—Guinea pigs were anesthetized with ether, and a 3-ml blood sample was withdrawn from each animal by cardiac puncture into heparinized syringes. To remove serum proteins, the blood cells were washed three successive times with 10-ml volumes of normal saline followed by 600×*g* centrifugation. The washed, packed red cells were resuspended in 40 ml of normal saline. Propylene glycol (0.5 ml) was added to the control cell suspensions, and 1 mg of I in 0.5 ml of propylene glycol was added to the I-treated cell suspensions.

The cell suspensions were mixed by inversion and incubated 1 hr at 37°. Cells were removed from suspension by centrifugation and washed three times with normal saline to remove unbound I. After washing, the hapten-modified or sham-treated red cells were resuspended to 3 ml with saline and injected into the marginal ear vein of the animal of cell origin. Guinea pigs were anesthetized intramuscularly with 50 mg of ketamine[5]/kg and 0.5 mg of chlorpromazine[6]/kg prior to injection.

Sensitization—Sensitization of all animals to I was attempted 2 weeks after haptenated or sham-treated red cell injection by topical application of 1 mg of I to the dorsal neck skin of ~5 cm^2. Animals were sensitized to dinitrochlorobenzene[7] by applying 2 μl of a 50% acetone solution of dinitrochlorobenzene to the dorsal surface of the left ear.

Skin Testing—Hair was removed from the abdominal skin with small animal clippers[8]. Test sites, ~1.5 × 1.5 cm, were delineated with a felt-tip pen[9], and 5 μl of a test solution or vehicle was dropped from a syringe[10] onto the skin within the sites. Sites not visibly healed from previous testing were not used for retesting. The test sites were observed and scored for presence and intensity of erythema and edema at 24, 48, and 72 hr after testing, using the scoring system of Draize *et al.* (23) (Table I). To compare the intensity of the inflammation of the responding sites of treated and control animals, the degree of delayed-type hypersensitivity for each group to the individual test doses was calculated. The erythema scores of the responding animals in each group were summed and divided by the number of animals in the group responding at 24, 48, and 72 hr. The edema scores of each group were averaged similarly. The averaged erythema and edema scores were added to obtain a 24-, 48-, and 72-hr value. The final degree of delayed-type hypersensitivity for each group was obtained by averaging the 24-, 48-, and 72-hr summed values as in Table II. The score of 8 is the maximal degree of delayed-type hy-

[1] Camm Institute, Wayne, N.J.
[2] Ralston-Purina, St. Louis, Mo.
[3] Brinkmann Instruments, Westbury, N.Y.
[4] New England Nuclear, Boston, Mass.
[5] Ketaset, Bristol Myers Co., Syracuse, N.Y.
[6] Thorazine, Smith Kline & French Laboratories, Philadelphia, Pa.
[7] Aldrich, Milwaukee, Wis.
[8] Angra clippers, John Oster Manufacturing Co., Milwaukee, Wis.
[9] Markette, Crestwood, Wilkes-Barre, Pa.
[10] Hamilton.

persensitivity attainable. To skin test for dinitrochlorobenzene sensitivity, a drop of 0.5% dinitrochlorobenzene in olive oil was inuncted into the flank with a glass rod; the sites were observed for presence of erythema at 24 hr after testing.

Data Analysis—Differences in the number of experimental and control animals presenting erythema responses to pentadecylcatechol were analyzed using χ^2 statistics. The data for each test period were arranged in a 2 × 2, treatment by response, contingency table. A null hypothesis of equal proportionality was tested using one degree of freedom.

RESULTS AND DISCUSSION

Red blood cells were either pentadecylcatechol modified or sham treated *in vitro* as described and injected intravenously into the guinea pig of cell origin 2 weeks prior to attempted sensitization with I. Groups of animals given haptenated or sham-treated red cells received identical treatment throughout the study. Pentadecylcatechol-modified red cell-induced tolerance was duplicated in five separate experiments, using a total of 55 treated and 40 control animals, with similar results. Data from two of these experiments were selected for this paper.

Induction of Tolerance to I with Pentadecylcatechol-Modified Red Cells—Twenty-four naive guinea pigs were injected with autologous pentadecylcatechol-modified red cells, and a similar control group was given autologous sham-treated red cells intravenously. Sensitization of both groups to I was attempted 2 weeks after treatment. A third group of 10 guinea pigs (negative controls) was not pretreated or sensitized. Two weeks after attempted sensitization and at 2- or 4-week intervals thereafter until the conclusion of the study, all animals were skin tested simultaneously with 3, 1, and 0.3 μg of I and with acetone. Test sites not visibly healed were not used. Since no erythema or edema was observed on any acetone test site, the vehicle control data were omitted.

To represent the skin test responses of each group concisely, the percentage of the animals in each group presenting erythema on one or more of the three observation periods (24, 48, and 72 hr) to the three test doses is shown in Table III. Injection of hapten–red cell conjugates 2 weeks prior to attempted sensitization to I produced a high level of tolerance to skin test doses of I that persisted for 20 weeks. The frequency of reactivity of the control group to the 3 μg of I ranged from 70 to 100% throughout the study, while the percentage responding in the haptenated red cell-treated group ranged from 21 to 33%. The reactivity of the control group to 1 μg of I ranged from 63 to 83%, whereas 0–17% of the group treated with haptenated red cells were sensitive to this test dose. None of the treated animals ever reacted to 0.3 μg of I at any time during the study, while the sensitivity of the control group to this dose ranged from 4 to 46%. Tolerance to I did not appear to wane with time but was partially broken by the second attempt at sensitization after the 20th week. However, the sensitivity of the controls tended to decrease slowly after the 8-week test period and was not strengthened by the second sensitizing dose.

The sensitivity of the pentadecylcatechol-modified red cell-treated animals ranged from complete tolerance to moderate sensitivity, whereas the control group responses were moderate to extreme. The tolerance of seven of the treated animals was complete in that they never responded to any test dose of I during the entire study. Six of the treated animals responded only once during the study, and these responses were +1 erythema to 3 μg of I. Four treated animals responded on two skin test periods to 3 μg of I; these responses were mainly +1 erythema, but an occasional +2 erythema was observed. Two additional treated animals responded to 3 μg of I on four or five of the nine test periods; most of these responses were +2 erythema with rarely a +1 edema. The remaining five treated animals (20%) were not perceivably tolerant and responded to 3 μg of I on nearly every occasion. These animals also responded occasionally to 1 μg of I.

Overall, the data indicate that 80% of the treated group was tolerant to I from the 20 weeks following attempted sensitization to I. Testing of 10 naive guinea pigs with the three test solutions resulted in primary irritancy responses in two out of the 10 animals to 3 μg of I. Since this dose was found by others to be irritating in ~50% of naive animals tested, the occasional responses to this dose in the treated animals may represent false positive responses. Only two animals in the control group failed to respond to doses of <3 μg.

To compare the differences in sensitivity of the treated and control animals, the degree of delayed-type hypersensitivity for each group was calculated (Table IV). The sensitivity of the responding animals in the control group gradually declined after the 6-week test and was not increased by a second sensitizing dose at Week 20. The sensitivity of the responding animals in the treated group, on the other hand, gradually increased until the 14th week, and a second attempt at sensitization did not increase their sensitivity.

Statistical analysis of the data showed that at the 3-μg test level, significant differences existed between the treated and control groups

Table III—Occurrence of Erythema to 3-, 1-, and 0.3-μg Test Doses of Pentadecylcatechol in Pentadecylcatechol-Modified Red Cell-Treated and Sham-Treated Guinea Pigs

Treatment	Weeks[a]	Percent of Group with Definite Erythema Response to Test Doses of Pentadecylcatechol		
		3 μg	1 μg	0.3 μg
Hapten-modified red cell	2	33	0	0
Control	2	96	63	38
Hapten-modified red cell	4	33	0	0
Control	4	100	79	38
Hapten-modified red cell	6	21	4	0
Control	6	92	71	29
Hapten-modified red cell	8	21	8	0
Control	8	92	71	17
Hapten-modified red cell	10	29	8	0
Control	10	88	67	33
Hapten-modified red cell	14	33	17	4
Control	14	92	71	46
Hapten-modified red cell	16	26	13	0
Control	16	88	83	42
Hapten-modified red cell	20	26	9	0
Control	20	70	43	4
Hapten-modified red cell	24[b]	52	22	0
Control	24[b]	95	43	10

[a] Weeks after attempted sensitization to pentadecylcatechol. [b] A second sensitizing dose of pentadecylcatechol was given to both groups after the 20-week test period.

Table IV—Average Degree of Delayed-Type Hypersensitivity to Pentadecylcatechol-Modified or Sham-Treated Red Cells 2 Weeks prior to Attempted Sensitization to Pentadecylcatechol

Treatment	Weeks[a]	Average Degree of Delayed-Type Hypersensitivity to Test Doses of Pentadecylcatechol		
		3 μg	1 μg	0.3 μg
Hapten-modified red cell	2	0.8 (8/24)[b]	0.0 (0/24)	0.0 (0/24)
Control	2	3.4 (23/24)	2.0 (15/24)	0.8 (9/24)
Hapten-modified red cell	4	0.9 (8/24)	0.0 (0/24)	0.0 (0/24)
Control	4	3.5 (24/24)	2.1 (19/24)	0.8 (9/24)
Hapten-modified red cell	6	1.1 (5/24)	0.3 (1/24)	0.0 (0/24)
Control	6	3.8 (22/24)	2.1 (17/24)	0.3 (7/24)
Hapten-modified red cell	8	1.6 (5/24)	0.5 (2/24)	0.0 (0/24)
Control	8	2.8 (22/24)	1.8 (17/24)	0.9 (4/24)
Hapten-modified red cell	10	1.4 (7/24)	0.5 (2/24)	0.0 (0/24)
Control	10	2.2 (21/24)	1.5 (16/24)	0.7 (8/24)
Hapten-modified red cell	14	1.7 (8/24)	1.1 (4/24)	0.0 (0/24)
Control	14	2.5 (22/24)	1.2 (17/24)	0.8 (11/24)
Hapten-modified red cell	16	1.7 (6/23)	1.0 (3/23)	0.0 (0/23)
Control	16	1.7 (21/24)	1.3 (20/24)	0.6 (10/24)
Hapten-modified red cell	20[c]	1.3 (6/23)	1.2 (2/23)	0.0 (0/23)
Control	20[c]	2.1 (16/23)	0.8 (10/23)	0.7 (1/23)
Hapten-modified red cell	24	1.4 (12/23)	1.3 (5/23)	0.0 (0/23)
Control	24	1.7 (20/21)	0.6 (9/21)	0.7 (2/21)

[a] Weeks after attempted sensitization to pentadecylcatechol. [b] Number of responders per number tested in parentheses. [c] A second sensitizing dose was applied after this test.

Table V—Occurrence of Erythema Reactions to Pentadecylcatechol (I) and Its Olefinic Congeners in Pentadecylcatechol-Modified Red Cell-Treated and Sham-Treated Control Animals

Treatment	Weeks[a]	Percent of Group with Definite Erythema Response to Test Doses of I or Its Olefinic Congeners					
		I			Monoolefin,	Diolefin,	Triolefin,
		3 μg	1 μg	0.3 μg	3 μg	3 μg	3 μg
Pentadecylcatechol-modified red cell	2	23[b]	15	0	NT[c]	NT	NT
Control	2	88	25	0	NT	NT	NT
Pentadecylcatechol-modified red cell	4	15[b]	0	0	NT	NT	NT
Control	4	75	38	0	NT	NT	NT
Pentadecylcatechol-modified red cell	6	15[b]	NT	NT	15[d]	15[d]	15[d]
Control	6	100	NT	NT	100	100	100
Pentadecylcatechol-modified red cell	8	18[b]	NT	NT	18[b]	18[b]	36[b]
Control	8	88	NT	NT	88	100	100

[a] Weeks after attempted sensitization with pentadecylcatechol. [b] $p < 0.01$. [c] Not tested. [d] $p < 0.001$.

Table VI—Degree of Delayed-Type Hypersensitivity of Pentadecylcatechol-Modified (E) or Sham-Treated Control (C) Groups of Guinea Pigs to Pentadecylcatechol (I) and Its Olefinic Congeners

Treatment	Weeks[a]	Skin Test Substances					
		I			Monoolefin,	Diolefin,	Triolefin,
		3 μg	1 μg	0.3 μg	3 μg	3 μg	3 μg
E	2	1.5	0.3	0	NT[b]	NT	NT
C	2	1.7	1.0	0	NT	NT	NT
E	4	1.0	0.0	0	NT	NT	NT
C	4	1.0	0.8	0	NT	NT	NT
E	6	0.8	NT	NT	2.2	1.8	2.8
C	6	1.1	NT	NT	1.7	2.0	2.8
E	8	1.0	NT	NT	1.2	1.1	3.1
C	8	1.6	NT	NT	1.2	1.9	3.1

[a] Weeks after attempted sensitization. [b] Not tested.

throughout the entire 24 weeks. At the 1-μg dose level, significant differences were observed over the first 14 weeks. Loss of significance at the 20 and 24th weeks to the 1-μg test level apparently resulted from a loss of sensitivity in the control group rather than from a loss of tolerance in the experimental group. At the 0.3-μg dose level, the differences in tolerant and control groups were not consistently significant due to the paucity of responses in the control group.

To show that tolerance to I was caused by haptenated red cell treatment and not by lack of immunocompetence, all treated animals were given sensitizing doses of dinitrochlorobenzene after the initial skin test with I. The control guinea pigs were not given sensitizing doses and served as negative dinitrochlorobenzene controls. None of the control group responded to dinitrochlorobenzene whereas all of the treated tolerant animals developed sensitivity to it. Therefore, tolerance induced by pentadecylcatechol-modified red cell injection was immunologically specific.

Specificity of Pentadecylcatechol-Induced Sensitivity and Pentadecylcatechol-Modified Red Cell-Induced Tolerance—A group of 11 guinea pigs was given tolerizing injections of haptenated red cells, and a control group of eight was given sham-treated red cells. Sensitization of both groups to I was attempted 2 weeks later. Both groups were skin tested with 3-, 1-, and 0.3-μg doses of I and with vehicle at 2 and 4 weeks after attempted sensitization. At 6 and 8 weeks after attempted sensitization, each group was tested with 3 μg each of I and its mono-, di-, and triolefinic congeners. The frequency of erythema responses of these animals to the skin test doses are shown in Table V. Animals were scored as reactive if erythema was observed at least once during the 72-hr observation period after test substance application.

The degree of delayed-type hypersensitivity of treated and control groups to I and its congeners was calculated as previously described and is shown in Table VI. All control animals developed sensitivity to I and its three congeners, but the skin reactions to the different congeners were not of equal severity. The degree of delayed-type hypersensitivity produced by triolefin was two to three times that produced by I. The degree of delayed-type hypersensitivity decreased with increased saturation of the side chain. The order of allergenic potency was triolefin > diolefin > monoolefin > I. The haptenated red cell-treated pigs were highly tolerant to I and its congeners throughout the 8 weeks of study. The differences in sensitivity of the treated and control groups were highly significant ($p < 0.01$ or <0.001) at the 3-μg level of I and its congeners throughout the 8 weeks. Differences were not significant at the 1- and 0.3-μg doses of I because of lack of a high degree of sensitivity of control animals. Over 80% of the treated animals in this experiment were tolerant, which confirms the results of the previous study. Again, two animals in the treated group (15%) did not develop tolerance.

Table VII—Determination of Radioactivity Associated with Intact Tritiated Pentadecylcatechol Red Cell Conjugates, Red Cell Membrane Ghosts, and Red Cell Sap[a]

	Tritium, μCi		Percent of Total Activity		Percent of Incorporated Activity	
Unincorporated (incubation supernate)	3.29		47.0			
First wash (supernate)	0.28		4.0			
Second wash (supernate)	0.15		2.5			
Third wash (supernate)	0.09		1.9			
Total unincorporated		3.81		55.4		
Incorporated						
Lysate of whole cells	0.24		3.4		12.7	
Wash of ghosts	0.12		1.7		6.3	
Total cell sap		0.36		5.1		19.0
Red cell ghost		1.53		21.95		80.9
Total recovered activity		5.70		82.45		99.9

[a] Total activity added to suspension was 7 μCi.

Determination of Membrane-Associated Pentadecylcatechol—Tritium-labeled pentadecylcatechol red cell conjugates were prepared to determine the percentage of I that became bound to the red cell and the localization of I (membrane associated or cell sap associated). Cell suspensions were incubated with 1 mg of I (7 μCi of tritiated pentadecylcatechol). After removal of the incubation supernate (unincorporated activity) followed by three successive washes, aliquots of the conjugates were used to determine the radioactivity incorporated into the intact cells or they were lysed with hypotonic solution, and the activity associated with the red cell membrane ghosts and cell sap was determined.

The determinations were made on four separate cell suspensions with essentially the same results. The results of a typical assay are shown in Table VII. Twenty-seven percent of the activity (equivalent to 270 μg of I) became associated with the red cell. Fifty-five percent of the activity was in the incubation mixture, and the remaining 17.5% was believed to have been retained in the incubation vessel. Approximately 81% of the red cell-incorporated activity (equivalent to 219 μg of I) was associated

with the red cell membrane. The remaining 19% was associated with the red cell sap and represented ~51 μg of I.

It was reported that membrane-associated urushiol or I (red blood cell or lymphocyte membrane associated) will induce blastogenesis of peripheral blood lymphocytes *in vitro* (24). Urushiol was shown to be highly soluble in, but not covalently bound to, the cell membrane since the urushiol was not removed from the membrane with aqueous washes but was removable with dimethyl sulfoxide. Haptenated membranes were shown to induce contact sensitivity to picryl chloride *in vitro* when administered subcutaneously (25), while intravenous administration of the haptenated membranes induced specific immunological tolerance. Thus, the administration route of the membrane-associated hapten appears to be important in determining whether contact sensitivity or tolerance will result.

Since haptenated membranes can induce hapten-specific tolerance as well as contact sensitivity, it is unlikely that the haptenated membrane is the tolerogen and sensitizer. The membrane probably serves as a physiological vehicle that carries the lipophilic substance until the immune system intercepts the hapten. Whether contact sensitivity or tolerance results probably depends on the prevalence of the interceptor cell type (macrophage, Langerhans cell, T-lymphocyte, B-lymphocyte) that initially reacts with the hapten. The route of hapten administration likely would favor a higher incidence of interaction of the hapten with one of these cell types.

REFERENCES

(1) W. L. Epstein, *Cutis*, **13**, 544 (1974).

(2) S. Billets, J. C. Craig, M. D. Corbett, and J. F. Vickery, *Phytochemistry*, **15**, 533 (1976).

(3) M. D. Corbett and S. Billets, *J. Pharm. Sci.*, **64**, 1715 (1975).

(4) W. F. Symes and C. R. Dawson, *J. Am. Chem. Soc.*, **76**, 2959 (1954).

(5) S. V. Sunthankar and C. R. Dawson, *ibid.*, **76**, 5070 (1954).

(6) K. H. Markiewitz and C. R. Dawson, *J. Org. Chem.*, **30**, 1610 (1965).

(7) M. Gross, H. Baer, and H. M. Fales, *Phytochemistry*, **14**, 2263 (1975).

(8) R. A. Johnson, H. Baer, C. A. Kirkpatrick, C. R. Dawson, and R. G. Khurana, *J. Allergy Clin. Immunol.*, **49**, 27 (1972).

(9) H. Keil, D. Wasserman, and C. R. Dawson, *Science*, **102**, 279 (1954).

(10) A. M. Kligman, *Arch. Dermatol.*, **78**, 47 (1958).

(11) W. L. Epstein, H. Baer, C. R. Dawson, and R. G. Khurana, *ibid.*, **109**, 356 (1974).

(12) H. Baer and M. Hooton, in "Animal Models in Dermatology," H. I. Maibach, Ed., Churchill Livingstone, New York, N.Y., 1975, pp. 53–55.

(13) R. T. Bowser and H. Baer, *J. Immunol.*, **91**, 791 (1963).

(14) H. Baer, M. L. Hooton, C. R. Dawson, and D. I. Lerner, *J. Invest. Dermatol.*, **69**, 215 (1977).

(15) D. Lerner, *Diss. Abstr. Int. B*, **37**(7), 3419 (1977).

(16) U. Forsiem, L. Klareskog, U. M. Tjernlund, and P. A. Peterson, *Acta Derm. Venereol. Suppl.*, **79**, 37 (1978).

(17) M. W. Chase, *Proc. Soc. Exp. Biol. Med.*, **61**, 257 (1946).

(18) I. Silberberg-Sinakin and R. L. Baer, *Prog. Allergy*, **24**, 268 (1978).

(19) J. R. Battisto and J. Miller, *Proc. Soc. Exp. Biol. Med.*, **111**, 111 (1962).

(20) J. R. Battisto and B. R. Bloom, *Nature (London)*, **212**, 156 (1966).

(21) H. N. Claman, S. D. Miller, and J. W. Moorehead, *Cold Spring Harbor Symp. Quant. Biol.*, **41**, 105 (1977).

(22) J. C. Craig, C. W. Waller, S. Billets, and M. A. ElSohly, *J. Pharm. Sci.*, **67**, 483 (1978).

(23) H. H. Draize, G. Woodward, and H. D. Calvery, *J. Pharmacol. Exp. Ther.*, **82**, 377 (1944).

(24) V. S. Byers, W. L. Epstein, N. Castagnoli, and H. Baer, *J. Clin. Invest.*, **64**, 1437 (1979).

(25) M. I. Greene, S. M. Sugimoto, and B. Benacerraf, *J. Immunol.*, **120**, 1604 (1978).

ACKNOWLEDGMENTS

The authors thank Ms. Mary Rungeling for technical assistance in preparation of this manuscript.

Laser Raman Investigation of Pharmaceutical Solids: Griseofulvin and Its Solvates

BARBARA A. BOLTON and PARAS N. PRASAD ˣ

Received July 24, 1980, from the *Department of Chemistry, State University of New York at Buffalo, Buffalo, NY 14214.* Accepted for publication December 16, 1980.

Abstract □ Laser Raman spectroscopy is convenient for characterizing griseofulvin solvates and investigating solute–solvent interactions and desolvation. The spectra of both lattice and intramolecular vibrations were monitored. A new solvate of griseofulvin with bromoform was characterized by Raman spectroscopy. A temperature-dependence study of the solvates of griseofulvin with chloroform, bromoform, and benzene revealed no phase transformation or chemical change. In the benzene solvate, only weak Van der Waals interactions existed between the solute and solvent. However, in solvates with chloroform and bromoform, a weak hydrogen bonding existed between the proton of the solvent and the C=O group of the benzofuran ring in griseofulvin. Examination of desolvation in these solvates revealed that the crystal did not go through any intermediate structure during desolvation. As the solvent molecule escaped, the lattice reverted to the structure of unsolvated griseofulvin.

Keyphrases □ Griseofulvin—unsolvated and solvate forms, laser Raman spectroscopy, physicochemical stability, desolvation □ Spectroscopy, laser Raman—investigation of griseofulvin and its solvates, physicochemical stability, desolvation □ Pharmaceutical solids, polymorphic—griseofulvin and its solvates, investigation using laser Raman spectroscopy, physicochemical stability, desolvation □ Antifungal agents—griseofulvin and its solvates, investigation with laser Raman spectroscopy, physicochemical stability, desolvation

Many drugs in the solid state exhibit polymorphism, in which the same compound exists in several crystalline modifications at the same temperature (1, 2). Interest in polymorphism stems from the fact that different crystalline modifications of the same drug have different physical and, in some cases, chemical properties that may be a serious consideration in the manufacture of the dosage form (1, 2). For example, differences in the solubility of polymorphic forms can cause serious bioavailability problems.

0022-3549/81/0700-0789$01.00/0