In Vitro Studies of Poison Oak Immunity

II. EFFECT OF URUSHIOL ANALOGUES
ON THE HUMAN IN VITRO RESPONSE

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A B S T R A C T Studies were performed to ascertain the effect of urushiol analogues on the in vitro lymphocyte blastogenesis elicited by urushiol in peripheral blood lymphocytes taken from individuals sensitized to poison oak or ivy. Urushiol is a mixture of alkylcatechols composed of a catechol ring coupled to mono-, di-, or tri-unsaturated C-15 or C-17 carbon side chains. Each of these two moieties, catechol ring and side chain, was tested for its role in eliciting reactivity. Analogues tested represented the catechol ring (3-methylcatechol), the mono- or di-unsaturated side chain (oleic or linoleic acid), and the saturated side chain coupled to a catechol ring (pentadecylcatechol), a blocked catechol ring (heptadecylveratrole), or a resorcinol (pentadecylresorcinol). Urushiol with a blocked catechol ring (urushiol dimethyl ether) was also included.

Of these, only pentadecylcatechol evoked reactivity in sensitized lymphocytes, and this reactivity was only a fraction of that evoked by urushiol. This suggested that the system has some requirement for the side chain, and that the catechol ring is critical for reactivity. This was further investigated by testing the ability of some of these analogues to inhibit urushiol-specific blastogenesis. No inhibition was noted with compounds bearing the saturated side chain with modified ring structures (pentadecylresorcinol and heptadecylveratrole). However, both 3-methylcatechol and pentadecylcatechol (at equimolar concentrations) blocked reactivity. The results of our experiments suggested that although both the side chain and the catechol ring are required for reactivity, the latter is most critical. Unsaturation in the side chain is important for maximal reactivity because the saturated catechols were only partially as active as the urushiol oil. There may be a greater dose requirement for the catechol ring than for the side chain.

INTRODUCTION

In a previous report (1) we demonstrated that urushiol-specific blastogenesis can be elicited from cultured peripheral blood lymphocytes taken from individuals spontaneously or experimentally sensitized against poison oak. The reactive cell is a T lymphocyte which requires an accessory cell from the T-depleted population (probably a macrophage) to produce blastogenesis. The response has a narrow dose range, and supraoptimal doses of urushiol produce a sharp inhibition of response. Urushiol can be introduced into cultures on heterologous, homologous, or autologous erythrocyte membranes or on autologous lymphocytes with equal efficiency. Although the alkylcatechols that compose urushiol concentrate into membranes and remain firmly associated through aqueous washes, the association does not appear to be irreversible because the majority of the radioactivity introduced with a radiolabeled hapten can be removed with organic solvents (1).

Studies on T-cell mediated immunity to haptens have suggested that covalent linkage between the hapten and a carrier macromolecule (protein) is required to evoke a blastogenesis response in vitro (2). The carrier protein is thought to associate with a macrophage-like cell which may either present the surface-bound hapten as such or, alternatively, may process the hapten internally before presentation to a reactive T cell (3). Urushiol is a mixture of catechols that are readily oxidized to electrophilic o-quinones thought to be capable of spontaneously alkylating nucleophilic functionalities present in cell membrane proteins. It has been suggested that this mechanism of binding is responsible for the potent in vivo sensitizing properties of urushiol (4). However, our in vitro

studies indicated that the great majority of the urushiol that produced blastogenesis was reversibly bound to the cell membranes used to introduce it into culture (1). This suggests that the catechol ring is not as capable of spontaneous auto-oxidation and covalent bond formation as originally suggested. To determine if there was a requirement for the catechol ring for reactivity and to evaluate the influence of the catechol ring and unsaturated side chains on biological reactivity, the ability of urushiol analogues to evoke blastogenesis or to block urushiol-induced blastogenesis has been studied.

METHODS

Compounds. Urushiol oil was provided by Dr. H. Baer (Bureau of Biologics, Food and Drug Administration, Bethesda, Md.) and was extracted from poison oak and described previously as the "Mississippi lot" (5).

The side-chain saturated compound 3-*n*-pentadecylcatechol (PDC)[1] was synthesized by Dr. John Kurtz and provided by Dr. W. Acres of the Letterman General Hospital, San Francisco, Calif. This compound was stored at −70°C under nitrogen. The C-17 di-*O*-methyl ether 3-*n*-heptadecylveratrole (HDV) was synthesized as an intermediate in the preparation of 3-*n*-heptadecylcatechol (HDC) (1). Synthetic details will be published later.[2] The PDC positional isomer 5-*n*-pentadecylresorcinol (PDR) and 3-methylcatechol were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Linoleic acid was obtained from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. Oleic acid was obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.

[3H]PDC (30 mCi/mM) was obtained from Dr. H. Baer and was purified by thin-layer chromatography. [3H]HDV (5 mCi/mM) was prepared by ^3H_2O decomposition of the trialkylboran product obtained from 1-heptadec-2-enyl-2, 3-dimethoxybenzene, and diborane.[2] *O*-Demethylation of [3H]HDV with BBr_3 gave [3H]HDC. Urushiol dimethyl ether (UDE) was obtained in essentially quantitative yield by treatment of an ethereal solution of urushiol (100 mg) with a 20-fold excess of diazomethane under N_2 at 4°C for 4 d. After adding acetic acid (1 ml) to decompose excess diazomethane and washing the ether layer twice with aqueous K_2CO_3, the ether was dried over Na_2SO_4, and the solvent was removed under vacuum to provide an oily residue (100 mg). The electron ionization mass spectrum displayed ions at M^+ 373 (ene), 372 (diene), and 370 (triene) in the ratio of 1:3:13. The 80 MHz NMR spectrum displayed the expected signals including two singlets at δ 3.56 and 3.59 ppm for the methyl ether protons.

Preparation of membranes carrying urushiol and/or urushiol analogues. Urushiol was added to cultures on erythrocyte membrane carriers as previously described (1). Urushiol analogues or analogue-urushiol mixtures were also

added to cultures on erythrocyte membranes such that one batch of membranes carried both urushiol and the analogue. Because 3-methylcatechol is water soluble it was added directly to cultures.

Analogue-urushiol membranes were prepared by resuspending 10^{10} human erythrocytes (RBC) in 4.5 ml of Hanks' balanced salt solution (HBSS). To this was added 0.5 ml of dimethyl sulfoxide (DMSO) that contained 0.5 mg of analogue, and, after incubation for 30 min at room temperature, 40 ml of sterile water was added and the mixture was centrifuged at 20,000 g for 30 min, and the membranes were resuspended in water. After two additional water washes, the membranes were suspended in 4.5 ml of water to which 0.5 mg of urushiol in 0.5 ml of DMSO had been added. After a second 30-min incubation at room temperature, the membranes were washed as before and resuspended in HBSS at the appropriate concentration and added to cultures.

Three series of studies were carried out with radiolabeled compounds to quantitate the amount of membrane-bound analogue and urushiol, which were thus introduced to the cultures. In the first series, [3H]HDC or [3H]HDV was mixed with the unlabeled compound and then added in 0.5 ml of DMSO to 4.5 ml of HBSS that contained 10^{10} RBC. After a 30-min incubation period at room temperature and three water washes as before, the membranes were resuspended in 2.0 ml of water, and an aliquot was dissolved in NCS solubilizer (Amersham Corp., Arlington Heights, Ill.) and counted as previously described (1). In the second series of experiments, radiolabeled HDC or HDV was added at different concentrations in 0.5 ml of DMSO to HBSS that contained 10^{10} RBC. After incubation and three water washes, the membranes were resuspended again in 4.5 ml of HBSS and unlabeled HDC, HDV, or urushiol was added at various concentrations in 0.5 ml of DMSO. After incubation and water washes the membranes were again resuspended and an aliquot was digested and counted as before. In the third series, the sequence was reversed; the unlabeled material was added, and then after three washes the labeled material was added. It was assumed for purposes of calculation that all of the analogue and/or urushiol added to the RBC membranes was taken up into the membranes. In one set of experiments, radiolabeled PDC was used instead of radiolabeled HDC because of scarcity of materials.

Blastogenesis assay. Lymphocyte blastogenesis was assayed by the methods previously described (1). Peripheral blood lymphocytes from a reactive donor were separated and cultured in microwells in RPMI 1640 with 10% autologous serum added. In most cases, the analogues and/or urushiol on RBC membranes were added at various concentrations to the wells. The 3-methylcatechol was added directly to the cultures either at fixed concentrations or at concentrations such that the molarity of 3-methylcatechol in each culture represented the same as that of the urushiol added on RBC membranes. Concanavalin A-induced blastogenesis was carried out as previously described (1).

Statistical analysis. Data from multiple experiments were compared in Table III as to the blastogenesis produced by either urushiol or one of the analogues. These data could not appropriately be analyzed by a standard *t* test because the standard deviations varied from experiment to experiment and between urushiol- and analogue-treated triplicate samples. Instead a generalized Welch test was used, and calculated as follows:

$$t_{4n} = \frac{\Delta}{s} \sqrt{\frac{3}{2n}} ,$$

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[1] *Abbreviations used in this paper*: DMSO, dimethyl sulfoxide; HBSS, Hanks' balanced salt solution; HDC, 3-*n*-heptadecylcatechol; HDV, 3-*n*-heptadecylveratrole; PDC, 3-*n*-pentadecylcatechol; PDR, 5-*n*-pentadecylresorcinol; RBC, erythrocyte(s); UDE, urushiol dimethyl ether.

[2] Jacob, P., D. Liberato, and N. Castagnoli, Jr. Manuscript in preparation.

when

$$\Delta = \sum \bar{x}_{urushiol} - \sum \bar{x}_{analogue},$$

and

$$s = \sqrt{\frac{\sum sd^2_{urushiol} + \sum sd^2_{analogue}}{2n}},$$

and

$$n = \text{number of experiments.}$$

RESULTS

Binding of compounds to RBC membrane carriers.
The structures of the compounds used in these studies
are shown in Fig. 1. All except 3-methylcatechol are
lipophilic and are soluble in cell membranes. RBC
membranes provided an efficient and convenient
method of adding urushiol and analogues to cultures
of lymphocytes (1). The same procedure was used to
add the analogues to cultured lymphocytes in this study.
RBC membranes were prepared either with the
analogues alone or were first treated with analogue
at the same concentration as was used for urushiol,
washed, and then treated with urushiol. To examine
the amount of urushiol and the amount of the analogues
that bind to the carrier membrane, and thus is intro-
duced into the culture, a series of binding/competition
experiments was carried out with radiolabeled PDC,
HDC, or HDV. Because of limited materials avail-
able, [³H]PDC and [³H]HDC were used interchange-
ably; for purposes of the binding studies, both were

considered as models of urushiol. [³H]HDV served as
an urushiol analogue which is unlikely to form
covalent bonds to macromolecules because it cannot
be oxidized to an electrophilic quinone species.

The results of the first series of experiments are
shown in Table I. Different amounts of unlabeled
urushiol, HDC, or HDV were mixed with radiolabeled
HDC, PDC, or HDV, to determine whether urushiol
and analogues could be incorporated into the RBC
membrane under the conditions used for presenta-
tion of these compounds to lymphocyte cultures. The
results of these experiments are as follows: (*a*) Mem-
branes (from 10^{10} RBC) can accommodate up to 5 mg of
PDC and 2.5 mg of urushiol without significant loss
of uptake of [³H]HDC. Amounts higher than this
began to show decreased recovery of counts. This is
probably not related to the binding capacity of the
membranes but rather to the fact that the membranes
become sticky and cannot be completely removed
from their containers. (*b*) Behavior of the various
analogues in terms of simple uptake suggests that the
dominant structural feature determining uptake is the
lipophilic side chain. HDV becomes incorporated to
about the same extent as HDC, and one does not dis-
place the other. (*c*) Results of the second and third
series of experiments described in Methods, in which
labeled and unlabeled compounds were bound
sequentially, were essentially the same as if the com-
pounds were premixed, as in series 1.

*Blastogenesis response stimulated by urushiol or
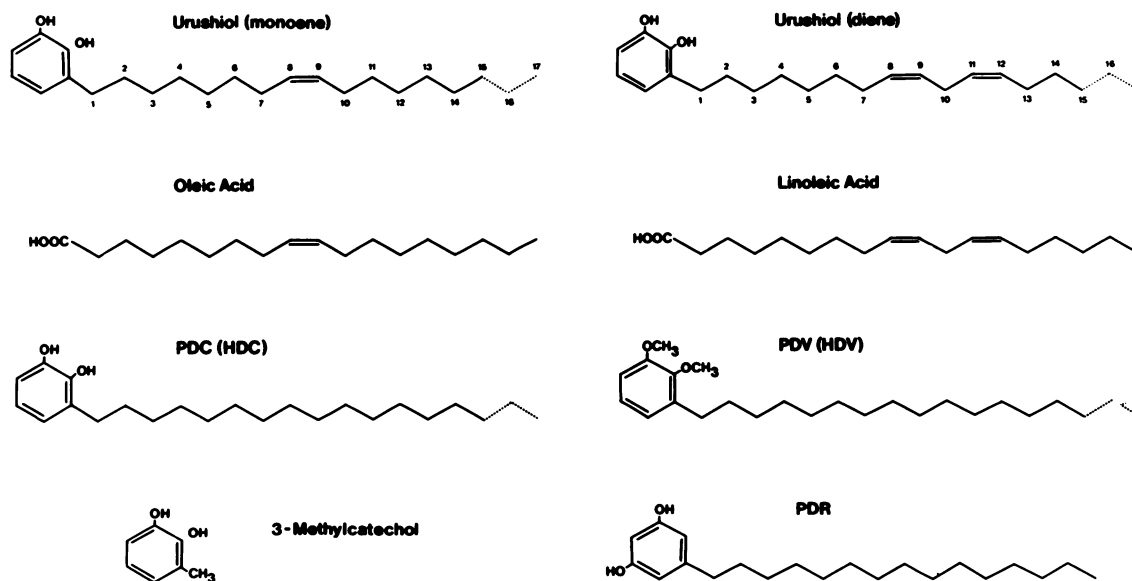analogues.* Peripheral blood lymphocytes from poison

FIGURE 1   Structure of urushiol components that contain either C-15 or C-17 side chains and
analogues used in this study.

TABLE I
Binding Capacity of RBC Membranes for Urushiol and Its Analogues

Labeled compound	Unlabeled compound	Counts bound	Labeled compound bound
		$cpm \pm SEM \times 10^3$	mg
0.500 mg [³H]HDC*	None	2,606±20	0.420
	5.00 mg PDC	2,921±12	0.471
	0.50 mg PDC	2,610±26	0.421
	0.50 mg Urushiol	2,595±26	0.419
0.063 mg [³H]HDC*	None	321±5	0.052
	0.630 mg PDC	348±3	0.056
	0.063 mg PDC	339±7	0.055
	0.630 mg Urushiol	349±3	0.053
	0.063 mg Urushiol	329±3	0.053
0.050 mg [³H]HDC*	None	287±5	0.046
	5.00 mg PDC	272±9	0.044
	0.50 mg PDC	340±8	0.055
	0.50 mg Urushiol	347±1	0.056
0.500 mg [³H]HDV‡	None	299±6	0.344
	5.00 mg PDC	207±4	0.234
	0.50 mg PDC	302±2	0.347
	2.500 mg Urushiol	224±2	0.257
	1.250 mg Urushiol	255±1	0.293
	0.625 mg Urushiol	268±6	0.308

* 6.2 × 10⁶ cpm/mg [³H]HDC.
‡ 8.7 × 10⁵ cpm/mg [³H]HDV.

oak-sensitized individuals were stimulated with a range of concentrations of RBC-bound urushiol and the analogues listed in Tables II and III. Table II depicts the results of urushiol and six analogues tested on urushiol-sensitized lymphocytes from a single donor in one experiment, and Table III summarizes the results from multiple experiments. Treatment with doses of urushiol as low as 0.008 μg/culture produced strong responses. The response to PDC also was positive in about the same dose range and was always less than one-half that of urushiol. HDC also reacted in the same dose range as PDC, and the intensity of re-activity was somewhat lower. None of the other compounds, including PDR, linoleic acid, 3-methylcatechol, or HDV, produced blastogenesis. Cultures that contained both linoleic acid and 3-methylcatechol also were nonresponsive.

To determine if the absence of HDV activity was a result of the lack of an oxidizable catechol moiety or the absence of unsaturation in the side chain, UDE activity was examined. UDE, which like HDV has the blocked catechol ring but unlike HDV retains the unsaturated side chain, was completely inactive. These results suggest that the hapten must contain both the

TABLE II
Comparison of Blastogenesis Response Induced by Urushiol or Urushiol Analogues in Urushiol-reactive Lymphocytes

Compound tested*	[¹⁴C]Thymidine uptake‡	Percent urushiol reaction§
	$cpm \pm SEM$	%
Control‖	442±59	0
Urushiol	3,315±271	100
PDC	1,182±200	26
HDC	507±82	2
HDV	517±58	3
PDR	411±66	−1
UDE	443±118	0
Linoleic acid	396±57	−2

* Urushiol and its analogues presented on RBC membrane carriers. 0.5 mg of compound was added to 10¹⁰ RBC in 10% DMSO–HBSS. Four concentrations of compound were tested ranging from 0.008 to 1.0 μg/well. Peak reactivity was found at 0.04 μg/well, which is the value reported in this table.
‡ Cultures of 4 × 10⁵ lymphocytes pulsed with [¹⁴C]thymidine on day 4 of culture and harvested on day 5.
§ Percent urushiol reaction = $\dfrac{cpm_{analogue} - cpm_{control}}{cpm_{urushiol} - cpm_{control}}$.
‖ Lymphocytes without antigen.

TABLE III
Summary of Experiments Comparing Blastogenesis Response with Urushiol
and Related Analogues in Urushiol-reactive Donor

Compound tested	No. of experiments	Maximum cpm	Stimulation index*	Percent urushiol response‡
		mean ±SEM	mean ±SEM	%
Urushiol	15	4,898±735§	10.0±1.5	100
PDC	7	2,346±1,130¶	3.6±0.6	23
HDC	2	917±96¶	2.2±0.4	15
HDV	2	780±110¶	1.3±0.2	5
PDR	2	710±252¶	1.2±0.1	4
UDE	2	445±115¶	1.4±0.1	4
Linoleic acid	4	603±111¶	1.2±0.2	1
3-methylcatechol	5	454±125¶	0.7±0.1	−7
3-methylcatechol + linoleic acid	1	874**	0.8	−10

* Stimulation index is calculated by dividing counts per minute of cultures that contain antigen by counts per minute of cultures that contain no antigen (control). Values are calculated for each experiment, then averaged.

‡ Percent urushiol response = $cpm_{analogue} - cpm_{control}/cpm_{urushiol} - cpm_{control}$ calculated for each experiment, and then averaged.

§ Represents the mean counts per minute of the triplicate samples from each experiment, ±standard error of the mean between experiments.

|| Average value of control cultures in these 15 experiments = 596±373 cpm.

¶ $P < 0.001$ when compared with urushiol response, analyzed by the generalized Welch test.

** $P < 0.01$ when compared with urushiol response, analyzed by the generalized Welch test.

aliphatic side chain and the catechol moiety to elicit blastogenesis, and that an unsaturated side chain is required for maximal activity.

Effect of analogues on urushiol-stimulated blastogenesis. To study the effect of HDV and PDR on the urushiol response, RBC membranes were treated with analogue alone, urushiol alone, or analogue and urushiol. The treated membranes were added at various concentrations to cultures of sensitized lymphocytes. To simplify calculations, it was assumed that 100% of the compounds added to the RBC membranes are membrane bound. The justification for this assumption came from the binding studies with radiolabeled HDC and HDV shown in Table I. Therefore, cultures either contained urushiol on RBC membranes, analogues on RBC membranes, or equimolar concentrations of urushiol plus analogue. The effects of the analogue on urushiol-mediated blastogenesis are shown in Fig. 2A. Both HDV and PDR produced marked amplification of the response. This amplification effect was extended through all the dose ranges. There was no inhibitory effect. The effect of 3-methylcatechol on the urushiol response was also tested. Because 3-methylcatechol is water soluble, it would not be expected to bind to RBC membranes. Therefore, urushiol was added to cultures on RBC membranes as before, and 3-methylcatechol was added to each culture at a concentration equimolar

to that of the urushiol. As can be seen in Fig. 2B, this compound inhibited the urushiol response over a wide dose range.

This inhibition was further investigated in a second set of experiments shown in Fig. 3. Four sets of cultures were prepared, each containing increasing doses of RBC-bound urushiol. To each of the sets was added either 1,667 pmol of 3-methylcatechol, 833 pmol of 3-methylcatechol, 417 pmol of 3-methylcatechol, or no analogue. Controls included concanavalin A added to each set of cultures. All three concentrations of 3-methylcatechol produced inhibition of the urushiol response in a dose-dependent fashion. This inhibition was antigen specific because the concanavalin A response was not affected by either urushiol or 3-methylcatechol.

The effect of PDC on urushiol-induced blastogenesis was complex because the apparent effect of PDC was to inhibit the urushiol reactivity produced by optimal urushiol concentrations (Figs. 4 and 5). Dose-response analysis, however, established that the entire urushiol response curve had been shifted to the left, such that high levels of blastogenesis were being produced at levels of urushiol which normally were completely inactive. For example (Fig. 4), when PDC or urushiol alone was added to cultures, the maximal response occurred at concentrations of ≅200 ng of alkylcatechol per culture. However, if the membranes used to stimu-
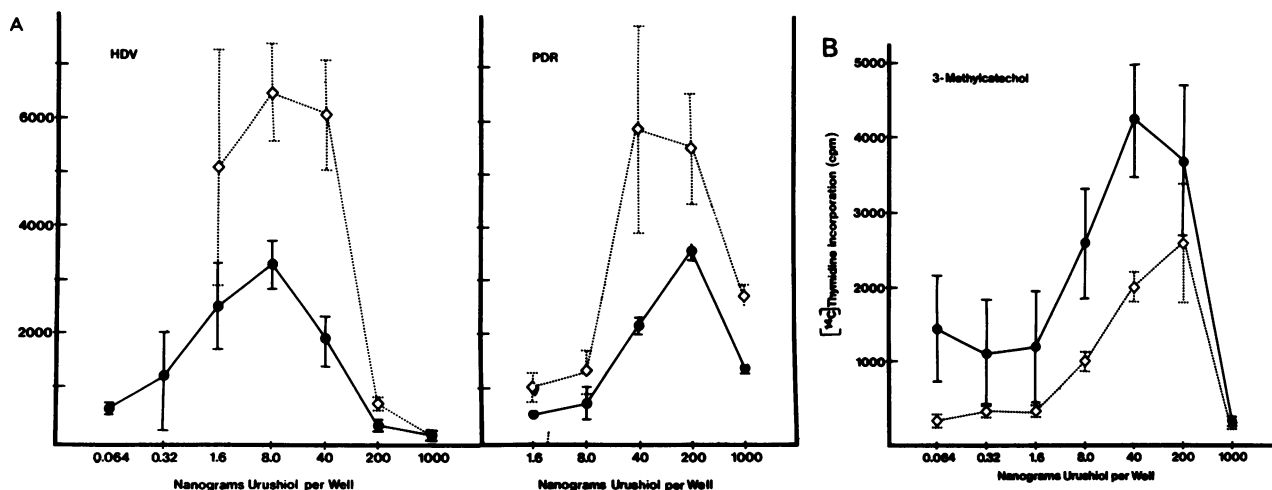
FIGURE 2   Effect of urushiol analogues on the urushiol-mediated lymphocyte blastogenesis response. RBC membranes (10^{10}) were treated with 0.5 mg of either urushiol (●) or of urushiol analogue followed by 0.5 mg of urushiol (◇). These membrane preparations were added in variable amounts to cultures of urushiol-reactive lymphocytes, and the blastogenesis response was plotted against the amount of urushiol or analogue per culture. Six replicate cultures provide data for each point, and 40 ng of urushiol corresponds to 8×10^3 RBC membranes per culture. (A) Effects of equimolar concentrations of either HPV or PDR added together with urushiol on RBC membranes. (B) Effect of equimolar concentrations of 3-methylcatechol on urushiol-induced blastogenesis. Increasing numbers of RBC membranes carrying urushiol were added to cultures. To each of these cultures, 3-methylcatechol was also added in an amount equimolar to that of the urushiol.
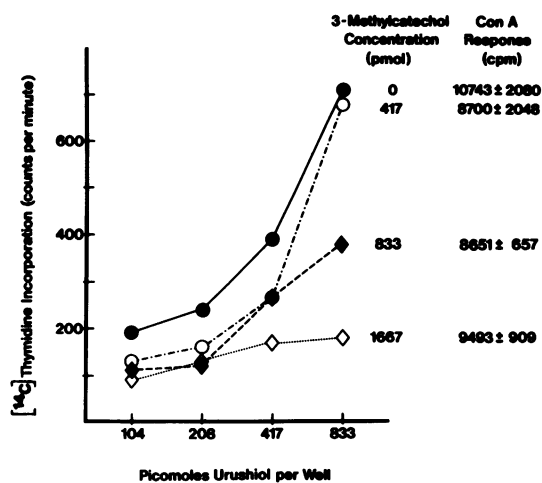

FIGURE 3   The effect of three different concentrations of 3-methylcatechol on the response of sensitized lymphocytes to urushiol or concanavalin A (Con A). Four sets of cultures were prepared each containing increasing doses of RBC-urushiol. To each set was added either 1,667 pmol of 3-methylcatechol (◇), 833 pmol of 3-methylcatechol (◆), 417 pmol of 3-methylcatechol (○), or no 3-methylcatechol (●). Controls included concanavalin A added to each set of cultures. The concanavalin A response in cultures that contained the appropriate concentration of 3-methylcatechol but no urushiol is depicted opposite each 3-methylcatechol concentration.

late blastogenesis carried a 5:1 mixture of PDC and urushiol, then a peak reaction, which was almost maximal, occurred at 40 ng of urushiol per culture. Because this amount of urushiol in the absence of PDC is inactive, it would appear that PDC is acting to shift the urushiol activity curve to the left.

Titration experiments were carried out in which the ratio of PDC to urushiol was varied from 1:1 through 100:1, and the results (Fig. 5) indicate that at ratios of PDC:urushiol of 50:1 the left-shift-effect was still present, although the intensity of response was diminished. This shift in dose requirement is interesting because if the total catechol required for peak reactivity at each PDC:urushiol ratio is calculated, it is in the range of 100–200 ng/culture, comparable to that required for urushiol alone. It thus appears that PDC can substitute for up to 98% of the urushiol required to produce blastogenesis. This left shift was observed with both HDC and PDC, but with no other analogue tested.

DISCUSSION

Our studies were designed to investigate the nature of antigen presentation and the specificity involved in the cellular immune response to the hapten urushiol. A series of analogues of urushiol was tested to determine
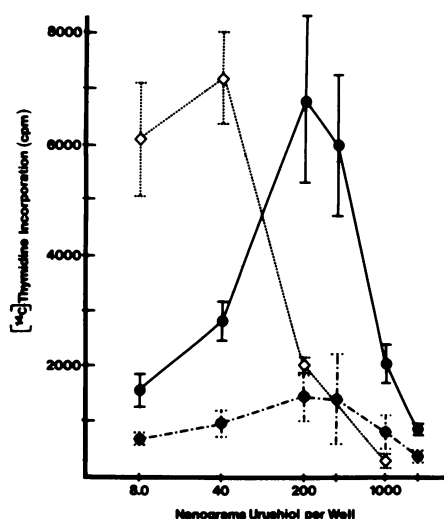
FIGURE 4 Effect of PDC on the urushiol response. RBC membranes were treated with PDC (0.5 mg/10^{10} RBC), washed in water, and then treated with urushiol (0.5 mg, ◇). The blastogenesis response of these membranes is compared with that produced by RBC membranes treated with either urushiol alone (● —— ●) or PDC alone (● – – – ●).

the ability of these compounds to stimulate or inhibit blastogenesis in urushiol-sensitive lymphocytes, both alone and mixed with urushiol.

Both urushiol and PDC induce peak levels of blastogenesis within the same narrow dose range, but the urushiol response is greater than that of PDC. These results suggest that the unsaturated side chain plays a
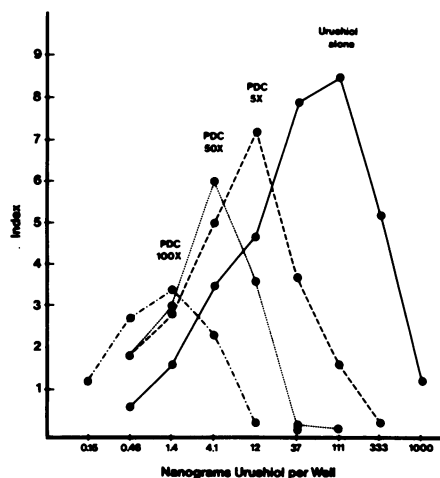

FIGURE 5 Blastogenesis response induced by RBC membranes treated with PDC and then urushiol. RBC membranes were treated with PDC at doses ranging from 0.5 to 0.05 mg/10^{10} RBC membranes. After washing in water, aliquots of these membrane preparations were treated with 0.05 or 0.005 mg urushiol, washed, and then added at varying amounts to the cultures.

significant role in the urushiol-induced blastogenic response. Human in vivo studies have also established that the di-unsaturated urushiol carries the majority of the reactivity (6). The di-unsaturated side chain must be coupled to a catechol to produce a blastogenic reaction in vitro. Compounds mimicking the di-unsaturated side chain (linoleic acid) or the mono-unsaturated side chain (oleic acid) of urushiol were inactive in vitro. Also inactive was 3-methylcatechol, as was an equimolar mixture of 3-methylcatechol and linoleic acid. The catechol moiety is essential for reactivity because neither PDR nor HDV was active. These compounds share the saturated side chain with HDC and PDC, but have ring structures unable to generate reactive electrophilic species. The necessity for a catechol ring is further supported by the finding that UDE, incapable of forming the electrophilic o-quinone, is also inactive, even though this species retains the unsaturated side-chain moiety. Four of the urushiol analogues, PDC, PDR, HDV, and 3-methylcatechol, were tested for their effect on urushiol-induced blastogenesis. Three of these analogues, PDC, PDR, and HDV, share long C-15- or C-17-saturated side chains coupled to ring structures. HDV and PDR, however, do not have the reactive catechol function. These two compounds amplified the response to urushiol. The dose range of this amplified response was the same as that of urushiol alone.

The two compounds with active catechol rings, PDC and 3-methylcatechol, both produced inhibition of the urushiol response. This inhibition was studied further with 3-methylcatechol. It was shown that as the concentration of this analogue was increased, the inhibitory effect on the urushiol response became more profound. The inhibition appeared antigen specific because blastogenesis induced with the mitogen concanavalin A was not affected by inhibitory concentrations of 3-methylcatechol. This series of experiments suggests that the catechol ring of urushiol interacts with a specific receptor. This receptor may be antigen specific, recognizing the catechol ring as its epitope, or relatively less specific in which it simply allows a covalent bond to form joining the urushiol hapten to a carrier.

Our experiments also suggest that the RBC carriers used in these experiments to introduce urushiol into the culture preparations may not be the ultimate carrier for the hapten urushiol. That is, the urushiol may be passed to some accessory cell which may oxidize it to the quinone and lead to covalent macromolecular adducts. This antigen may then be presented to sensitized T cells. These experiments do indicate that urushiol is initially presented to the immune machinery as the catechol because, otherwise, 3-methylcatechol should not block the reaction.

One of the analogues, PDC, produced a small blastogenic response when used alone to stimulate cultures. When added with urushiol it produced a leftward shift in the urushiol blastogenic response curve. The dose of urushiol required for optimal blastogenic response was reduced 50-fold. The noncatechol analogues did not produce this shift nor did 3-methylcatechol. The effect was not simply additive because the response elicited by urushiol or PDC alone at these concentrations was significantly lower. Regardless of the ratio, the optimal response of the PDC/urushiol mixture occurred at about the same total alkylcatechol concentration as that required for optimal reactivity to PDC or urushiol alone. The PDC shift was observed consistently when the PDC and urushiol were on the same carrier RBC membrane. This effect may be explained in several ways. PDC simply may be incorporated into the membrane of the presenter cell and thereby stabilize the specific antigen being presented to the blastogenically reactive T cell. In this case, PDC would be expected to be a nonspecific enhancer of antigen presentation and T-cell blastogenesis. An alternative possibility is that the requirement for the catechol ring is $\cong 50$ times greater than the requirement for the di-unsaturated side chain, and this need can be met by either PDC or urushiol. Because of its low lipid solubility, 3-methylcatechol would not meet this requirement.

The role of the unsaturated side chain in the hapten remains unclear. There is a marked loss of reactivity both in vivo (6) and in vitro if individuals immunized against the native oil that contains the unsaturated side chains are tested with the saturated side-chain compound, PDC. This suggests that a T-cell receptor may be directed against the side chain. However because of their lipophilic nature, the alkylcatechols should orient themselves in a membrane with the catechol ring near the surface and the hydrophobic chain buried deep in the membrane. It is thus difficult to conceive how the side chain could move out of the lipid membrane across aqueous media and trigger a specific response. One mechanism may be a membrane vessicle (7). Alternatively, specificity of the blastogeni-

cally active T cell may be against the catechol ring, and the increased reactivity with the unsaturated side-chain compounds could be a result of an altered presentation of the ring.

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