Radioimmunoassay for Phorbol Esters Using Rabbit Antisera against Phorbol Succinate

Armen H. Tashjian, Jr., Galina Wolfson, and Clare W. Fearon

ABSTRACT

The phorbol nucleus was succinylated and then conjugated to bovine albumin using dicyclohexylcarbodiimide. Rabbitts given injections of the conjugate developed antibodies which rose in titer progressively with repeated immunization. By the ninth bleeding, the binding of one antiserum, diluted 1:15,000, was saturated with about 10 nm [3H]phorbol-12,13-dibutyrate ([3H]-PDBU) and had an average association constant, Ks, of 2.6 x 10^8 M^-1. The serological specificity of the antiserum was characterized by examining the inhibition of the [3H]PDBU-anti-phorbol succinate immune system by 18 phorbol-related compounds. The specificities of antibodies from two rabbits tested in detail were qualitatively similar. The rank order of inhibitory activity for certain phorbol-related compounds was PDBU (concentration of inhibitor required to give 50% inhibition of PDBU binding (IC50) = 7.6 nm) > phorbol-13-acetate [IC50 = 8.2 nm] > phorbol-12,13-dibenzoate > 4-β-phorbol [IC50 = 124 nm] > phorbol-12,13-diacetate > phorbol-12-myristate-13-acetate [IC50 = 184 nm] > phorbol-13,20-diacetate > phorbol-12-acetate [IC50 = 2300 nm]. The following compounds showed no detectable serological activity: mezerein, 4-O-methylphorbol-12-myristate-13-acetate, ingenol, 4-α-phorbol, teleocidin B, and dihydroteleocidin B. These results indicated that the 4-β-phorbol nucleus was required for serological activity, that esterification of the C-13 position with benzoate, acetate, or butyrate enhanced the immunoreactivity of 4-β-phorbol, and that among the phorbol-related compounds examined there was no direct relationship between serological activity and biological potency as tumor promoters. Using the [3H]PDBU-anti-phorbol succinate immune system, we measured the concentrations of immunoreactive phorbol-related material in crude mixtures such as croton oil and performed pharmacokinetic studies in rats given PDBU s.c.

INTRODUCTION

Immunoassays for chemical carcinogens and for covalently modified carcinogen target molecules, often purine or pyrimidine bases in DNA, are now being used to be used in experimental carcinogenesis and in epidemiological studies (1, 7, 10, 12). The sensitivity and specificity of current immunological methods allow detection of modified DNA bases that occur at low frequency (about 700 O6-ethyl-2'-deoxyguanosine molecules per diploid genome) or even in single cells (1). To date, antibodies against the important class of tumor promoters, known as phorbol esters (3, 11), have not been described, and the major methods used to measure phorbol-related molecules have been isolation and chemical detection. In this report, we describe the production of antibodies against phorbol succinate in rabbits, the serological specificity of these antibodies, and their use to develop a radioimmunoassay for phorbol-related compounds.

MATERIALS AND METHODS

Materials. The following phorbol-related compounds were purchased from LC Services Corp. (Woburn, MA): P-13,20-DA, DPB, PDA, P-13-A, P-12-A, 4-α-PHR, PMA, 4-β-PHR, PTA, ingenol, PDBU, and mezerein. The following were purchased from Sigma Chemical Co. (St. Louis, MO): P-13,20-DA, P-20-O-20-Deo-MA, 4-α-PHR, PDBU, PDBZ, 4-β-PHR, PMA, 4-O-methyl-PMA, croton oil, diolein, and retinoid acid. All esters had the β-phorbol nucleus unless otherwise indicated. All compounds were dissolved in dimethyl sulfoxide to make fresh stock solutions (1 to 25 mg/ml). Diolein was dissolved in methanol. Stock solutions were stored at 20°. Teleocidin B, dihydroteleocidin B, and goat anti-rabbit IgG were generous gifts from Dr. Lawrence Levine, Brandeis University, Waltham, MA.

Bovine albumin (4 times crystallized) was from ICN Pharmaceuticals (Cleveland, OH). Succinic anhydride was from Sigma, and N,N′-dicyclohexylcarbodiimide and N-hydroxysuccinimide were from Pierce Chemical Co. (Rockford, IL).

The [3H]PDBU used was [20-3H]PDBU, (specific activity, 12.2 Ci/mmol; New England Nuclear, Boston, MA).

Preparation of Immunogen and Immunization. Phorbol succinate was prepared as follows. Phorbol was crystallized from croton oil and recrystallized from H2O:acetone as described (4). Phorbol (40 mg) and succinic anhydride (20 mg, recrystallized from chloroform) were incubated in 0.7 ml dimethylformamide at 22° for 24 hr (9). Water (1 ml) was added to the reaction mixture, and the solvent was removed by rotary evaporation at 42°. The residue was suspended in 1 ml H2O and extracted 3 times with 1 ml of ethyl acetate. Analysis of the ethyl acetate fraction on thin-layer chromatography (Sil G/UV254) revealed 2 products. The major spot (Rf 0.24, methylene chloride:acetone:acetic acid (3:1:0.1)) represented >80% of the material. A minor spot migrated at Rf 0.44 in the same solvent system. Both products were acidic, as revealed by reaction with bromocresol green. On the basis of the reactivities of the hydroxyl groups, on chromatographic behavior, and on conditions for synthesis of the corresponding phorbol acetate esters (9), we conclude that the major product was phorbol-13-monosuccinate and the minor...
component was phorbol-12,13-disuccinate. The chromatographic characteristics of the products were unchanged upon treatment with 30% acetic acid for 16 hr at 22°C, suggesting that there was no esterification at C-20 (9). In addition, the major component was isolated and treated with 0.17% perchloric acid in methanol for 16 hr at 23°C. By direct chemical ionization mass spectrometry, performed by Dr. Vernon Reinhold, the structure was determined to be phorbol-13-monosuccinate methyl ester.

Two immunogens were prepared by coupling the N-hydroxysuccinimide ester of phorbol succinate to bovine albumin. For Conjugate I (PS-BSA-I), phorbol succinate (44 μmol) was reacted with dicyclohexylcarbodiimide (10 mg) and N-hydroxysuccinimide (15 mg) in 1.5 ml acetonitrile for 16 hr at 4°C with stirring. The precipitate was removed by centrifugation, and the solvent evaporated with a stream of nitrogen. The derivatized phorbol succinate was incubated with bovine albumin (10 mg) in 0.1 M NaHCO₃ for 24 hr at 4°C and then dialyzed against 3 changes of potassium phosphate (15 mM), NaCl (150 mM), and bovine albumin (1 mg/ml), pH 7.5. By including [³H]phorbol during the preparation of phorbol succinate, we determined that there were 11 phorbols per bovine albumin molecule in Conjugate I.

For Conjugate II (PS-BSA-II), phorbol succinate (69 μmol) was esterified with N-hydroxysuccinimide by reacting it with dicyclohexylcarbodiimide (25 mg) and N-hydroxysuccinimide (40 mg) in 300 μl dimethylformamide for 16 hr at 4°C. This material (54 μmol) was coupled to 4 mg bovine albumin in 5 ml sodium phosphate (50 mM):NaCl (150 mM) (pH 7.5) for 16 hr at 4°C and then dialyzed against sodium phosphate (20 mM):NaCl (75 mM):bovine albumin (1 mg/ml), pH 7.5. The number of phorbols per albumin molecule was not determined for Conjugate II.

For immunization, PS-BSA was diluted with 0.9% NaCl solution to a final concentration of 600 μg protein/ml for Conjugate I and to 100 μg protein/ml for Conjugate II. The conjugate was emulsified with an equal volume of complete Freund's adjuvant, and a total of about 60 μg of Conjugate I or 10 μg of Conjugate II was injected i.d. in 20 to 24 sites on the shaved back of each 2.5-month-old female New Zealand White rabbit. The blood was allowed to clot in glass tubes at room temperature, and the serum was collected by centrifugation at 1800 x g for 20 min at 4°C.

Serum was assayed for immunoreactivity at volumes of 50 and 100 μl/assay tube in competition for [³H]PDBU binding to rabbit anti-phorbol succinate. Control tubes lacking rabbit antiserum revealed no binding of [³H]PDBU or interference in the radioimmunoassay by rat serum at volumes up to 100 μl/tube. The amount of phorbol-related immunoreactivity in serum (expressed as immunoreactive PDBU) was calculated from the PDBU standard curve.

**RESULTS**

**Binding of [³H]PDBU by Serum from Immunized Rabbits.** Four rabbits were given repeated injections of the phorbol succinate immunogens. The serum from all 4 injected rabbits bound [³H]PDBU. Final dilutions of 1:500 to 1:20,000 of serum from the immunized rabbits bound progressively decreasing amounts of [³H]PDBU, and the complexes were coprecipitated with goat anti-rabbit IgG (data not shown). At low dilution, up to 80% of the tracer added (about 10,000 cpm, 1.5 nm) was bound to antibody. Serum from 2 nonimmunized control rabbits did not bind [³H]PDBU even at high concentrations (final dilutions of 1:100).

As characteristic of antibody, the titer of the binding material in rabbit serum increased as immunization was continued (Chart 1). The results are shown for Rabbits Ra-74 and Ra-76. There was progressively more [³H]PDBU bound from Bleeding 2 (B-2) to Bleeding 8 (B-8). Similar results were obtained with Rabbits Ra-75 and Ra-77 (data not shown).

Binding of incremental additions of [³H]PDBU by rabbit antiserum Ra-74B-9 (diluted 1:15,000) is presented as a reciprocal plot (Chart 2). The combining sites of the antibodies in Ra-74B-9 measured at this dilution had an average association constant, Kₐ, of 2.6 x 10⁸ M⁻¹ at 37°C.

**Serological Specificity.** Inhibition of [³H]PDBU binding to 2 different antisera (Ra-74B-6 and Ra-76B-6) by a number of phorbol-related compounds was examined. Results with antisera Ra-74B-6 are shown in Chart 3. The specificities of the 2

---

**CANCER RESEARCH VOL. 45 JANUARY 1985**
antiseras were similar for all compounds tested except for ingenol, which was examined only with Ra-74B-6. PDBU and PDBZ were strong inhibitors; 4-β-PHR and PMA were less potent inhibitors; while ingenol, mezerein, 4-O-methyl-PMA, and butyric acid showed little inhibition even at high concentrations. Results with additional inhibitors tested with Ra-74B-6 are shown in Chart 4. The rank order of inhibitory activity was

PDBU = P-13-A > PDBZ > 4-β-PHR > PDA > P-13.20-DA > P-12-A. Ingenol, butyric acid, 4-O-methyl-PMA, and mezerein showed little serological activity. 4-α-Phorbol showed no serological activity (>4600 nm). A summary of the inhibitory potencies, determined in separate experiments, of all the phorbol-related compounds tested is given in Table 1. Several generalizations are apparent: (a) the 4-β-PHR nucleus is required for serological activity. Other potent tumor promoters, such as the structurally different compounds teleocidin B and dihydroteleocidin B, showed essentially no serological activity in this immune system; (b) among phorbol-related compounds, there was no direct relationship between serological activity and potency as a tumor promoter. For example, PMA, a potent promoter, was 24 times less immunoreactive than PDBU, a weak tumor promoter; (c) using [3H]PDBU as tracer, esterification at C-13 with benzoate, isobutyrate, acetate, or butyrate enhanced the serological activity of 4-β-PHR. Thus, serological activity correlated with the structure of the immunogen.

We tested several phorbol-related compounds from 2 different commercial sources, LC Services and Sigma. At multiple concentrations, we found no significant (<10%) quantitative or qualitative serological differences between P13.20-DA, 4-α-PHR, PDBU, 4-β-PHR, or PMA obtained from the 2 suppliers (data not shown).

Two additional tests of serological specificity were performed, (a) Anti-phorbol succinate Ra-74B-6 (1:500) was incubated, under identical conditions, with [3H]PDBU or [methyl-3H]TRH. Sixty ± 2% (range) of the [3H]PDBU and only 1.4 ± 2.0% of the [methyl-3H]TRH was coprecipitated with goat anti-rabbit IgG. Thus, the antiphorbol did not bind nonspecifically another 3H-labeled tracer. (b) unlabeled PDBU, 4-β-PHR, PDBZ, and PDD were tested as potential inhibitors in another rabbit antibody immune system. At concentrations up to 200 ng/tube, none of the 4 phorbol-related compounds inhibited the human calcitonin-anti-human calcitonin immune system (8) under conditions in which the homologous antigen, human calcitonin, inhibited >85% at 2.5 ng/tube (data not shown). Therefore, these phorbol-related compounds are not nonspecific inhibitors of all rabbit immune systems.

Applications. Using the [3H]PDBU:anti-phorbol succinate immune system, a radioimmunoassay has been developed which can measure phorbol-related immunoreactivity in crude natural materials such as croton oil or in biological fluids such as serum after administration of phorbol esters to experimental animals.

The data in Chart 5 show a dilution curve of unfractionated

Charts. Inhibition of [3H]PDBU binding to rabbit antiserum Ra-74B-6 (diluted 1:500) by various phorbol-related compounds. The competitor (1 to 1,000 ng/tube) and [3H]PDBU (about 10,000 cpm/tube) were incubated with antiserum Ra-74B-6 for 60 min at 37°, and the mixtures were then coprecipitated with goat anti-rabbit IgG. Each point gives the mean value for duplicate tubes; bars, ranges. Binding of [3H]PDBU in the absence of unlabeled competitor was 60% of tracer added. ING, ingenol; Mez, mezerein.
**PHORBOL ESTER RADIOIMMUNOASSAY**

**Chart 4.** Inhibition of [³H]PDBU binding to rabbit antiserum Ra-74B-6 (diluted 1/500) by several phorbol-related compounds. Results of 2 separate experiments are shown; the binding of [³H]PDBU (11,000 and 13,000 cpm/tube) in the absence of unlabeled competitor was 53 to 57% of tracer added. Bars, range.

**Table 1**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDBU</td>
<td>7.6</td>
</tr>
<tr>
<td>P-13-A</td>
<td>8.2</td>
</tr>
<tr>
<td>DPB</td>
<td>16</td>
</tr>
<tr>
<td>PDBZ</td>
<td>50</td>
</tr>
<tr>
<td>4α,8-PhHR</td>
<td>124</td>
</tr>
<tr>
<td>PDA</td>
<td>141</td>
</tr>
<tr>
<td>PMA</td>
<td>184</td>
</tr>
<tr>
<td>P-20-O-Deo-MA</td>
<td>214</td>
</tr>
<tr>
<td>P-13,20-DA</td>
<td>595</td>
</tr>
<tr>
<td>P-12-A</td>
<td>2,300</td>
</tr>
<tr>
<td>PTA</td>
<td>&gt;340</td>
</tr>
<tr>
<td>7-Dehydroteolecin B</td>
<td>&gt;370</td>
</tr>
<tr>
<td>PDD</td>
<td>&gt;495</td>
</tr>
<tr>
<td>Dihydroteolecin B</td>
<td>&gt;736</td>
</tr>
<tr>
<td>Mizonan</td>
<td>&gt;2,500</td>
</tr>
<tr>
<td>4α-O-Methyl-PMA</td>
<td>&gt;2,500</td>
</tr>
<tr>
<td>4α-PhHR</td>
<td>&gt;4,600</td>
</tr>
<tr>
<td>Ingelosin</td>
<td>&gt;4,600</td>
</tr>
<tr>
<td>Benzoyl acid</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Diolen</td>
<td>&gt;5,000</td>
</tr>
</tbody>
</table>

IC₅₀, concentration of inhibitor required to give 50% inhibition of [³H]PDBU binding.

---

**Chart 5.** Inhibition of [³H]PDBU binding to rabbit antiserum Ra-74B-6 (diluted 1/500) by increasing volumes of unfractionated croton oil. Each point gives the mean value of duplicate tubes; bars, ranges. The binding of [³H]PDBU (7,600 cpm/tube) in the absence of croton oil was 68% of tracer added.

**Chart 6.** Measurement of phorbol ester-related immunoreactivity in the serum of rats given PDBU by s.c. injection. A, concentrations of immunoreactivity (expressed in PDBU equivalents [PDBU]) in serum from 10 min to 22 hr after injection of 200 µg PDBU s.c. at zero time. Each point gives the mean value of serum PDBU equivalents obtained from 2 rats; bars, ranges. Each serum sample was tested at 50 and 100 µl assay tube. B, 2 standard curves for PDBU performed in absence (O) and presence (C) of 100 µl of normal rat serum/tube. The antiserum was Ra-74B-6 (diluted 1:500), and each tube contained 10,000 cpm of [³H]PDBU. *, binding of [³H]PDBU in the absence of unlabeled PDBU.

CROTON OIL ADDED (ml)

**Table 6.** Measurement of phorbol ester-related immunoreactivity in the serum of rats given PDBU by s.c. injection. A, concentrations of immunoreactivity (expressed in PDBU equivalents [PDBU]) in serum from 10 min to 22 hr after injection of 200 µg PDBU s.c. at zero time. Each point gives the mean value of serum PDBU equivalents obtained from 2 rats; bars, ranges. Each serum sample was tested at 50 and 100 µl assay tube. B, 2 standard curves for PDBU performed in absence (O) and presence (C) of 100 µl of normal rat serum/tube. The antiserum was Ra-74B-6 (diluted 1:500), and each tube contained 10,000 cpm of [³H]PDBU. *, binding of [³H]PDBU in the absence of unlabeled PDBU.

The results shown in Chart 6 illustrate the use of the radioimmunooassay for pharmacokinetic studies. Rats were given PDBU by s.c. injection, and the immunoreactivity in serum was determined at intervals from 10 min to 22 hr after injection. The time course of the appearance of immunoreactivity in serum is shown in Chart 6A. Significant blood concentrations (about 50 ng/ml serum) were detected 10 min after injection, and the peak concentrations were observed between 2 and 4 hr after injection. By 12 hr after injection, no detectable immunoreactivity remained in serum. The data in Chart 6B show the standard curve for croton oil in competition for [³H]PDBU binding to antiserum Ra-74B-6. As little as 4 × 10⁻⁷ ml croton oil gave significant inhibition of [³H]PDBU binding. If all the immunoreactivity in croton oil behaved like PDBU, there would be about 2 mg of PDBU equivalents per ml of croton oil.

---

CANCER RESEARCH VOL. 45 JANUARY 1985
PDBU in the absence and the presence of 100 μl of normal rat serum and indicate that rat serum does not alter the competition of PDBU for reaction in the [3H]PDBU:anti-phorbol succinate immune system. In the absence of unlabeled PDBU, the binding of [3H]PDBU to antibody was unaffected by the presence of 100 μl of normal rat serum (data not shown). These findings demonstrate that there is nothing in normal rat serum that competes for [3H]PDBU binding to antibody or that interferes with the quantitation of exogenously administered PDBU.

**DISCUSSION**

Rabbits immunized with phorbol succinate conjugated to bovine albumin develop antibodies directed at that phorbol portion of the complex. Several kinds of evidence support this conclusion: (a) normal rabbit serum does not contain activity that binds [3H]PDBU; (b) the binding activity present in the serum of rabbits given injections of the phorbol succinate conjugate increased progressively in amount as the rabbits were given repeated injections (Chart 1), a characteristic of the immune response; (c) the product formed on incubation of [3H]PDBU and serum from injected rabbits was precipitated by goat anti-rabbit IgG; (d) the reaction between [3H]PDBU and the serum of immunized rabbits was inhibited competitively by relevant hapten but not by unrelated or irrelevant hapten (Charts 3 and 4; Table 1); (e) the high binding constant of rabbit anti-phorbol succinate (2.6 × 10⁸ M⁻¹) is comparable to that reported for other lipid-related antigens (5) such as prostaglandin F₂α (5.4 × 10⁹ M⁻¹), prostaglandin B₁ (4.8 × 10⁹ M⁻¹), and leukotriene D₄ (2.8 × 10⁹ M⁻¹).

As indicated by the results of competition experiments with a variety of phorbol-related molecules (Table 1), it is clearly evident that our anti-phorbol succinate detects molecules that both have (PDBU and PMA) and do not have (4-β-PHR) biological activity as tumor promoters. Thus, the antibodies currently in hand cannot be used to measure or predict the tumor-promoting activity of unknown mixtures. On the other hand, as has been successfully applied in the prostaglandin area (2, 5), it should be possible to separate phorbol-related molecules chromatographically into discrete fractions which can then be quantitated at high sensitivity (ng range) by radioimmunossay. In addition, it should now be possible to prepare purposely new phorbol immunogens in such a way that the serologically active site corresponds more closely to the structural determinants of biological activity. In the case of our phorbol succinate immunogen, we purposefully prepared a conjugate in which the 4-β-PHR nucleus would have been immunodominant over specific ester side chains.

Finally, the results of the preliminary in vitro experiment, illustrated in Chart 6, indicate that the immunoassay approach to measurement of phorbol-related molecules can be applied to crude mammalian body fluids such as blood. By using chromatographic methods to separate the different phorbol products or metabolites, or by developing more specific antisera, or both approaches, it should be possible to measure low concentrations of specific phorbol esters in body fluids and tissue extracts and even to detect these compounds at the level of the individual cell by immunohistochemical methods.

**ACKNOWLEDGMENTS**

The authors thank Edward F. Voelkel for expert assistance with the immunization procedures; Dr. Lawrence Levine, Brandeis University, Waltham, MA, for gifts of the goat anti-rabbit IgG serum, teleocin A, and dihydroteleocidin B; and Dr. Vernon Reinhold, Harvard Medical Area Mass Spectrometry Facility, for analysis of the phorbol derivative.

**REFERENCES**


Radioimmunoassay for Phorbol Esters Using Rabbit Antisera against Phorbol Succinate

Armen H. Tashjian, Jr., Galina Wolfson and Clare W. Fearon


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/45/1/103

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.