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Serum Antibody Production—An Invariable Consequence of Sensitization with Dinitrochlorobenzene[1] (38867)

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In the course of studies on the immune response of guinea pigs to 2,4-dinitrochlorobenzene (DNCB), the sera of animals that received a single dose of this contactant were screened for the presence of antibodies to the dinitrophenyl (DNP) determinant. It was soon apparent that, without exception, a humoral response was elicited in every guinea pig treated with DNCB. The constancy of this finding stood in marked contrast to the reports of virtually all other investigators of this question. In previous studies the appearance of serum antibodies after even more intensive sensitization with DNCB was a sporadic and restricted event, limited to but a fraction of the treated animals (1–4).

In view of this discrepancy we undertook further studies of the humoral response to DNCB in contact sensitization by means of an antigen-binding technique used in this laboratory. This method of antibody measurement is designed to register all immunoglobulins capable of binding antigen, irrespective of their capacity to form precipitable aggregates or mediate diverse biological activities (5).

The data detailed below demonstrate that antibody formation regularly accompanies the development of contact sensitivity to DNCB in guinea pigs.

Materials and methods. Guinea pigs. Hartley strain guinea pigs of both sexes weighing 400 ± 50 g at the start of the experiments were used. These animals are maintained as a closed stock at this Institute.

Contact sensitization by epicutaneously applied DNCB. A single dose (1 mg) was administered by dropping 10 drops (5 μl each) (Drummond Microcaps, Drummond Scientific, Broomall, PA) of 2% DNCB (Eastman Kodak Co., Rochester, NY) in ethyl alcohol on the clipped skin of the back. This product migrated as a single spot when chromatographed as in (6).

Contact sensitization by injection of DNCB. One hundred micrograms of DNCB incorporated in complete Freund's adjuvant (0.12 mg dry weight mycobacteria) were injected into the four footpads in a volume of 0.5 ml. The injection material was prepared by rapid mixing of 1 part of an 0.5% solution of DNCB in ethyl alcohol with 11 parts of sterile normal saline. The mixture was immediately incorporated into an equal volume of complete Freund's adjuvant (CFA) containing 0.5 mg of *M. butyricum* per ml (Difco Laboratories, Detroit, MI). Seven and 14 days after sensitization, skin tests were performed as in (7) by dropping 5 μl of a series of DNCB solutions in ethyl alcohol on the clipped skin of the back. The concentrations of DNCB (1:100, 1:300, 1:1000, 1:3000, and 1:10,000) were chosen so as to elicit both positive and negative reactions in all animals. Approximately 74 μg of DNCB were, therefore, applied with each series of dilutions. Nonsensitized guinea pigs were tested routinely along with the experimental animals to serve as toxicity controls. The skin reactions were inspected at 24 and 48 hr and the lowest concentrations that elicited a positive reaction were recorded. The degree of contact sensitivity is expressed as the geometric mean of the threshold dose for each group of guinea pigs.

Collection of sera. The animals were bled by cardiac puncture on days 7, 14, and 70 after the sensitization procedure. The sera were collected and stored at −20°C.

Serum antibody assays. Serum anti-DNP levels were estimated by the mABC assay.

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This assay involves the interaction of a large excess of antigen with the diluted test serum so as to favor the formation of Ag_2Ab as the only immune complex. By appropriate calculations the peak antigen-binding values are converted into antibody weight units. The procedure described in (5) was followed with but slight modifications.

Diluent. Phosphate-buffered saline (0.15 *M* NaCl, 0.05 *M* sodium phosphate, pH 7.3) containing 0.0125 *M* sodium ethylenediaminotetraacetate (PBS-E) was used as diluent.

Antigen. DNP·HSA conjugates were prepared by reacting human serum albumin (HSA, Behring Diagnostics, Inc., Woodbury, NY) with 2,4 dinitrobenzene sulfonic acid (DNBS) (Eastman Kodak Co., Rochester, NY) at alkaline pH as described in (8). The degree of conjugation was assessed by absorbancy readings at 360 nm and dry-weight determinations. By varying the reaction time of the protein–DNBS interaction, a series of DNP-HSA conjugates differing in hapten-protein ratios was prepared. Those hapten–protein conjugates bearing an average of seven DNP groups per mole of HSA did not precipitate upon addition of an equal volume of saturated ammonium sulfate (SAS), and were, therefore, used in the antibody-binding assays.

Iodination. The procedure of Masouredis *et al.* (9) was followed with minor modifications. A radioactive iodine solution was prepared by mixing ca. 10 mCi of carrier-free ^{125}I (Union Carbide, Tuxedo, NY) in 0.2 ml of 9.05 *M* phosphate buffer, pH 7.4, with 0.8 ml of 0.05 *M* iodine in 0.1 *M* potassium iodide. The radioactive iodine was added dropwise to 39 ml phosphate buffer that contained 10 mg of protein N and allowed to react at room temperature with continuous stirring for 30 min. The protein solution was then dialyzed extensively against several 4-liter changes of phosphate-buffered saline (0.15 *M* NaCl, 0.005 *M* sodium phosphate, pH 7.1–7.3). The specific activity of the ^{125}I-protein solutions varied from about 180 × 10^3 to 200 × 10^3 cpm/μg N or less than 0.01 atoms of iodine per molecule. More than 99% of the radioactivity in all preparations was precipitable in 20% trichloroacetic acid. The protein content of the antigens was checked by optical-density measurements in alkali at 290 and 360 nm. Radioactivity estimates were carried out in a Packard autogamma spectrometer equipped with a NaI crystal.

Antigen solutions. Variable amounts of cold DNP·HSA and PBS-E were added to the stock ^{125}I·DNP·HSA to obtain the desired specific activity (20–45 × 10^3 cpm per μg N) and protein content for the mABC assays. Further dilutions of the antigen were prepared as needed in PBS-E.

mABC procedure. Half-milliliter volumes of an appropriate serum dilution were mixed with 0.5 ml of four or five dilutions of the ^{125}I-antigen in 75 × 10-mm disposable glass tubes and incubated for 30 min at room temperature before adding 0.9 ml of SAS. After 30 min of further incubation at 4° the tubes were centrifuged and the precipitates washed three times with 1.0-ml vol of 45% SAS. The precipitates were finally resuspended in 0.3 ml of 0.1 *N* NaOH. The amount of specifically bound antigen was obtained by subtracting the blank values from the cpm in the precipitates formed with the sera of the treated animals. The blank values represented the counts obtained with individual sera taken from the same animals prior to sensitization.

As discussed in (5) the antibody content of the sera is calculated by multiplying the amount of specifically bound antigen/ml serum by the molecular weight ratio factor Ab/2Ag. For our calculations we assumed the molecular weight of guinea pig IgG to be 163,000 daltons as in (10) and the molecular weight of DNP_7HSA as 70,170 daltons. The antibody N content per ml of undiluted serum was calculated as:

$$\mu g\ DNP_7HSA\ N\ bound \times \frac{163{,}000}{2 \times 70{,}170} \times serum\ dilution \times 2$$

In addition to the normal sera which provided the baseline values, a reference anti-DNP serum was included in all experiments. This serum, raised by conventional immunization of guinea pigs with DNP-proteins, was

diluted to match the antibody content of the test sera and assayed as a check of the mABC procedure.

Results. Delayed skin response to DNCB. Numerous assays in many normal guinea pigs (toxicity controls) indicated that they seldom reacted to as much as 250 nmoles of DNCB applied to the skin. Larger doses of the hapten were required to elicit a positive response in the untreated animals. In contrast, guinea pigs sensitized to DNCB by epicutaneous application or injection reacted to much lower doses of the hapten 1 wk later and for several months thereafter.

The injection of DNCB in CFA was a more effective sensitizing procedure than the epicutaneous application. Not only was the threshold dose lower after injection of DNCB with CFA, but the resulting skin responses were grossly inflamed and necrotic. The results of the day 7 and 14 skin tests performed in groups of 20 guinea pigs that received 1 mg DNCB epicutaneously or 100 μg DNCB in CFA are summarized in Table I. The mean threshold dose varied from 19 to 109 nmoles DNCB. The injected animals, sensitized with only one-tenth of the amount applied to the skin, reacted to 19 and 25 nmoles in successive tests, or roughly to about one-half or one-sixth of the threshold doses required to elicit a positive response in the skin-painted animals.

TABLE I. Threshold Doses for 48-Hour Skin Responses in Guinea Pigs Primed with DNCB.[a]

Mode of sensitization	Days after sensitization	
	7	14
	Nmol of DNCB	
1 mg DNCB, epicutaneous	41.5	108.6
100 μg DNCB, injected with CFA	24.7	19.3

[a] Each value represents the mean for 20 guinea pigs.

The humoral antibody response to DNCB. As shown in Table II a single epicutaneous application or injection of DNCB evoked a humoral immune response in each of the 40 guinea pigs used in this experiment. Measurable antibody levels were detectable 7 days after sensitization and were maintained or even increased during a further observation period of 10 wk. The early responses were of similar intensity in all animals. However, the guinea pigs injected with DNCB in CFA possessed higher antibody levels at the time of the last bleeding.

Representative assays of sera from DNCB-sensitized animals are given in Table III together with assays of a 14-day reference specimen from guinea pigs injected with DNP·BGG. Inspection of the data in Table III reveals the following pertinent considerations. Estimates of the antibody content of the anti-DNP·BGG serum were virtually identical when the assays were performed with serum diluted 41- or 82-fold, as was anticipated from earlier findings with protein antigens (11).

More important, perhaps, is the observation that for both of the anti-DNP·BGG serum dilutions an input of only 7 μg of DNP_7HSA N sufficed to achieve values close to those representing saturation of all available antigen-combining sites (Table III). This was generally not the case with the sera of guinea pigs immunized by epicu-

TABLE II. Serum Anti-DNP Responses in Guinea Pigs Primed with DNCB.

Mode of sensitization	Days after sensitization		
	7	14	70
	μg anti-DNP N per ml serum[a]		
1 mg DNCB, epicutaneous	11.4 (2.3–26.8)	10.4 (2.2–26.1)	12.1 (2.2–25.1)
100 μg DNCB, injected with CFA	7.5 (1.7–19.7)	13.1 (4.4–24.4)	28.8 (9.7–60.1)

[a] Mean and range of values for 20 animals in each group except for day 70 in which there were 15 guinea pigs per group.

TABLE III. REPRESENTATIVE mABC ASSAYS.

Immunogen	Days Post-immunization	Serum dilution used for assay	^{125}I·DNP$_7$HSA added[a] (μg N)	^{125}I·DNP$_7$HSA bound (μg N)	Anti-DNP per ml undiluted serum (μg N)
DNCB 1 mg epicut.	7	1→6	16	0.03	
			24	0.43	
			32	1.16	
			40	1.04	15.3
DNCB 1 mg epicut.	14	1→6	16	0.27	
			24	0.52	
			32	0.92	
			48	0.93	12.9
DNCB 1 mg epicut.	70	1→6	16	0	
			24	0	
			32	0.29	
			40	0.86	
			48	0.75	11.2
DNCB 0.1 mg in CFA	7	1→6	16	0.59	
			24	0.80	
			32	1.02	
			48	1.01	14.1
DNCB 0.1 mg in CFA	14	1→6	16	0.79	
			24	1.27	
			32	1.23	
			40	1.23	17.3
DNCB 0.1 mg in CFA	70	1→6	16	0.89	
			24	1.00	
			32	3.40	
			40	3.73	
			48	3.77	52.2
$DNP_{31}BGG$ 0.1 mg in CFA	14	1→82	7	0.63	
			14	0.69	
			29	0.87	
			50	0.89	169
	14	1→41	7	1.24	
			14	1.42	
			29	1.70	
			50	1.82	173

[a] Specific activity = 21.4×10^3 cpm per μg N.

taneous application of DNCB. With these sera an input of 16 μg of antigen N often yielded very low values relative to the peak binding levels. With DNCB in CFA sera intermediate binding occurred with a low antigen input. These findings suggest that the immunoglobulins produced by the administration of DNCB possess a lower mean affinity for DNP_7HSA than those produced by immunization with DNP·BGG.

Discussion. The data presented in this report permit the conclusion that serum antibody formation invariably accompanies the development of contact sensitivity in guinea pigs to DNCB. Although the tabulated results apply to 40 animals, similar findings have been obtained in no fewer than 75 guinea pigs during the course of these studies. It is, therefore, clearly apparent that a single application of DNCB elicits an immune response characteristic of both the humoral and cell-mediated types. This finding provides substantive evidence for the statement by Chase that contact sensitizers always stimulate the antibody-synthesizing apparatus (12). The failure to observe consistent antibody production in many previous studies in which more prolonged and intensive sensitization procedures were employed is attributable to the antibody detection methods used by other investigators (1–4). In virtually every other study the methods utilized for antibody detection and measurement relied on the secondary properties of the immunoglobulins such as aggregate formation with antigen, complement activation, and local cutaneous anaphylaxis. As is well known, these procedures account for only a variable segment of the total antibody populations, and, as shown in (13), are poorly suited for the detection of the early humoral response. In other respects, the present data confirm previous reports in showing that the injection of DNCB in Freund's complete adjuvant results in higher serum antibody levels (Table II) (14). The values reported here for the threshold levels of DNCB required to elicit the delayed response fall within the range anticipated from the data of Godfrey and Baer (6). These investigators injected 1 mg of hapten in CFA and reported a minimal reactive skin dose of 4.8 nmoles 47 days after sensitization. With 100 μg of DNCB and correspondingly less CFA we observed a threshold level of 19 nmoles on day 14.

The present data are of further interest in showing that serum antibody production after a single application of DNCB is not transient but like contact sensitivity persists for an extended period. Moreover, the intensity of the two manifestations of the im-

mune response paralleled each other. Antibody levels were higher in those guinea pigs with greater dermal sensitivity (cf. Tables I and II).

The humoral response to DNCB now shown to be consistently initiated by the hapten, suggests that specific immunoglobulins may play a role in contact sensitivity akin to that of humoral antibody in tumor and allograft cell-mediated immunity (15). Recently published evidence also points toward the presence of blocking and deblocking serum factors after the administration of dinitrophenyl haptens in guinea pigs (16).

Summary. Antibodies to the dinitrophenyl determinant as estimated by a modified antigen-binding procedure have been found in the sera of all guinea pigs with contact sensitivity to DNCB. The humoral as well as the dermal responses were detectable within 7 days after immunization and persisted for at least 10 wk.

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