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The Humoral Response to DNCB: Its Detection by Different Techniques[1] (38861)

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We have recently demonstrated that a single treatment of guinea pigs with 2,4 dinitrochlorobenzene (DNCB) induces a humoral immune response as well as contact sensitivity in all animals (1). This finding suggests that specific antibody synthesis is a more universal concomitant of the delayed skin response than is generally acknowledged. The uniformity of our results was contrasted with the rather sporadic demonstration of serum antibodies in other reports, in many of which the response to more intensive sensitization procedures was sought in terms of secondary antibody properties (2–5).

In view of these disparate findings and their theoretical implications, it was important to ascertain whether the various methods used to detect the humoral response could account for the observed differences. We, therefore, decided to compare the relative efficacy of several antibody assays for the detection and estimation of serum immunoglobulins formed after the administration of DNCB.

Sera from DNCB-sensitive guinea pigs previously shown to contain anti-DNP antibodies by means of the modified antigen-binding capacity assay (mABC) (6) were, therefore, reevaluated by precipitin, passive cutaneous anaphylaxis, and passive hemolysis procedures. The anti-DNCB sera were also assayed by the ABC-33 method described by Farr (7). The two antigen-binding assays differ from each other in several important respects. The major differences stem from the admixture of a constant volume of the test serum in the mABC assay with several amounts of antigen in large excess. This approach minimizes the dissociation of the antigen–antibody complexes and facilitates the detection of immunoglobulins with low binding avidities.

The experiments described below demonstrate the ability of the mABC assay to detect low levels of serum antibodies which escape recognition by the other methods, including the ABC-33 procedure.

Materials and Methods. Sera. The sera which were reexamined in the present studies were obtained from guinea pigs sensitized by a single treatment with DNCB. All of them contained antibodies to the DNP determinant as estimated by the mABC assay and reported in the previous communication (1).

Sera used as reference or positive controls in the antibody assays represented 14-day bleedings of guinea pigs injected with 100 μg DNP–BGG in CFA. The anti-DNP content of these sera were also estimated by mABC assays as reported in (8).

Bleedings taken prior to immunization provided the background values and negative controls for the several assays. All sera had been stored at −20°C.

Passive cutaneous anaphylaxis (PCA). PCA was performed as in (9) with 0.1 ml of the test samples injected intradermally. Sera of untreated and DNCB-treated guinea pigs were not diluted. Sera of animals injected with DNP–BGG were diluted 50-fold in saline prior to injection. Four animals were used for each serum in individual groups for which the latent period was either 4, 21, or 96 hr. The challenge consisted of an intravenous injection of 1 ml of saline containing 5 mg Evans blue and 500 μg $DNP_{35}BSA$. The skin sites were inspected 30 min after challenge.

Passive hemolysis. These titrations were performed as in (10) with serum dilutions ranging from 1:5 to 1:160. To these dilutions in volumes of 0.5 ml, sheep erythrocytes

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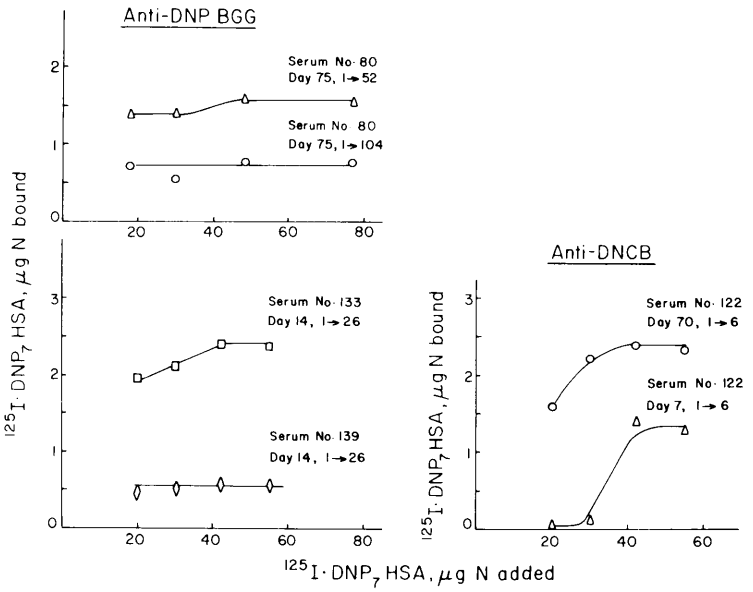

FIG. 1. Representative plots of the relationships between the amounts of antigen added and antigen bound in mABC assays with sera of guinea pigs immunized with $DNP_{31}BGG$ or with DNCB.

coated with $DNP_{35}BSA$ were added and the reaction mixtures incubated at 37° for 30 min. Fresh guinea pig serum, diluted 1:10 was then added and the tubes inspected for lysis over a 45-min period.

Quantitative precipitin analyses. These were performed with 0.5-ml vol of the undiluted sera to which were added several concentrations of $DNP_{37}HSA$ in phosphate-buffered saline, containing 0.125 *M* EDTA. The reaction mixtures were incubated for 60 min at 37° and 3 days in the cold. The precipitates were collected by centrifugation, washed twice, and analyzed for antibody content as in (11).

Double-diffusion analyses in gel were carried out in agar plates, pattern C, supplied by Hyland Labs., Costa Mesa, CA. The center wells received a solution of $DNP_{37}HSA$ containing 2 μg N per ml.

Antigen-binding assays. These were carried out as in (1, 6, 7). For the mABC procedure, 0.5 vol of the indicated serum dilutions were mixed with antigen levels usually in the range of 16–77 μg N antigen as shown in Fig. 1. The ABC-33 assays were performed with 2, 20, 200, and 400 ng of antigen N. The antigen used in both instances was $^{125}I \cdot DNP_7HSA$.

Results. Biological activity assays. Fourteen-day sera from five guinea pigs painted with DNCB and from another five injected with DNCB in CFA were assayed for PCA and hemolytic antibodies. Both assays included preimmunization and anti-DNP·BGG sera as controls. The antibody content of the anti-DNP·BGG serum was 63 μg Ab N/ml. The sera of the DNCB-treated guinea pigs contained from 2 to 44 μg Ab N/ml. The anti-DNP·BGG serum diluted 1:50 (0.13 μg N Ab/site) gave PCA reactions in three out of four guinea pigs after a 4-hr sensitization period and in all four animals after 21 hr of sensitization. This serum at a dilution of 1:160 also yielded total lysis of the DNP_{35}-BSA coated cells. None of the sera from the DNCB-treated animals or from the untreated controls sensitized the guinea pig skin for PCA. They also failed to induce lysis of DNP–BSA-coated sheep red blood cells, except for three sera of the DNCB-sensitized animals that induced only moderate lysis at the 1:5, 1:10, and 1:20 dilutions.

Quantitative precipitin assays. Pools of sera from DNCB-sensitized guinea pigs that were bled on days 7, 14, and 70, as well as before immunization, failed to yield visible precipitates upon incubation with DNP_{37}-

TABLE I. COMPARATIVE ANTIBODY WEIGHT ESTIMATES IN SERA OF GUINEA PIGS IMMUNIZED WITH DNP·BGG.

Micrograms antibody N per ml serum	
mABC assay	Precipitable anti-DNP
180	199
159	155
159	152
140	142
130	133
119	134
100	96
93	103
59	51

HSA. In contrast, assays of different pools of anti-DNP·BGG sera yielded very similar results when estimated by the mABC or quantitative precipitin procedures, as shown in Table I.

Immunodiffusion analyses. The photographs reproduced in Fig. 2 illustrate the results of immunodiffusion analyses carried out with six anti-DNCB sera. Each of the 7, 14, and 70-day bleedings formed a line of precipitation with $DNP_{37}HSA$ after 7-day incubation in the cold, as did the anti-DNP·BGG serum. The antibody content of the anti-DNCB sera as estimated by the mABC procedure ranged from 2 to 44 μg anti-DNP N/ml. The DNP·BGG antiserum used in these experiments contained 137 μg N Ab/ml. A reaction of identity was obtained between the lines formed by the anti-DNCB sera and those formed with the anti-DNP·BGG antiserum.

ABC-33 (Farr) assays. Twenty-two sera from guinea pigs that had been sensitized with DNCB were reexamined by the ABC-33 assay along with 10 sera from the preimmunized animals. None of these sera bound 33% of the antigen added in the assays. On this basis they would all be considered to lack anti-DNP antibodies. However, many of the sera from DNCB-treated animals did bind more antigen than did the preimmunization sera, as shown in Tables II and III. In these tables the percentage of antigen bound is indicated for assays with 2 and 20 ng N of DNP_7HSA and for initial serum concentrations of 1:6 and 1:10. In other experiments with antigen inputs of 200 and 400 ng N DNP_7HSA the percentage of antigen bound did not increase over that shown in the tables. Thus, none of these sera bound 33% of the antigen at the various serum dilutions with either 2, 20, 200, or 400 μg of DNP_7-HSA. In contrast, as shown in Table IV, 29 sera taken 14 days after the injection of DNP-BGG bound more than $\frac{1}{3}$ of either 20 or 200 ng DNP_7HSA. The binding capacity of these sera decreased markedly with the lower antigen input.

Discussion. The present data indicate that several immunologic assays other than the mABC and double diffusion in gel consistently failed to detect antibodies to the DNP determinant in the sera of DNCB-sensitized guinea pigs. If the assays used in these experiments had been limited to antigen binding by the ABC-33 procedure, specific precipitation in a fluid medium, passive cutaneous anaphylaxis, or immune hemolysis, the DNCB-sensitized guinea pigs would have been considered unresponsive in terms of humoral antibody synthesis. In marked contrast, 110 sera assayed by the mABC procedure in this and the previous study and 18 of these by double-diffusion analyses were shown to contain specific anti-DNP immunoglobulins. The essentially negative results obtained in PCA and passive lysis experiments could perhaps be accounted for in terms of the different functions subserved by the several immunoglobulin classes, e.g., IgG1 in the former case and IgG2 in the latter. These considerations, however, are hardly adequate. On the basis of the antibody levels estimated with the mABC approach (2–44 μg anti-DNP N per ml), the undiluted sera used in the PCA assays, and the dilutions used for demonstrating passive lysis should have contained adequate quantities of the appropriate immunoglobulins to yield positive results with these two techniques. Their failure to do so stems from the fact that these methods are unsuitable for comparative antibody estimations with sera of low avidity for antigen which are frequently obtained early in the immune response (9, 12). Similar qualifications apply to the use of precipitin analyses with sera of relatively low

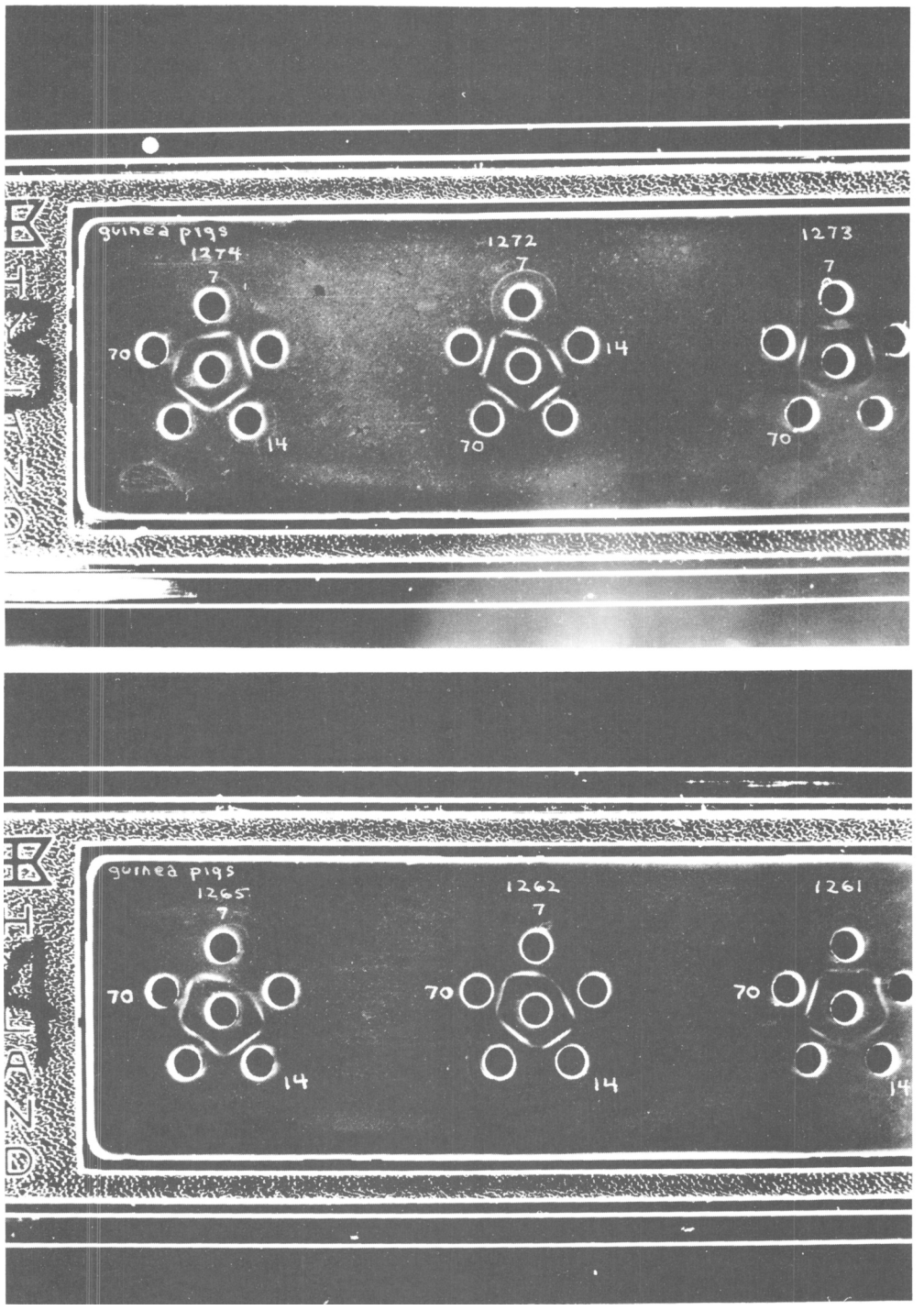

FIG. 2. Immunoplates showing lines of identity between precipitates formed with $DNP_{37}HSA$ and sera of guinea pigs immunized with $DNP_{62}BGG$ and with DNCB. The numbers in the peripheral wells indicate day of bleeding. Unlabeled peripheral wells received the anti-DNP-BGG serum. All central wells were filled with a solution of $DNP_{37}HSA$ contaning 2 μg N per ml. The sera in plate 3 were obtained after epicutaneous application of DNCB. The sera in plate 4 were obtained after the injection of DNCB in CFA.

TABLE II. COMPARATIVE ESTIMATES OF ANTIGEN-BINDING CAPACITY IN 14-DAY SERA BY THE mABC AND FARR ASSAYS.

Immunogen	Guinea pig number	mABC estimates μg anti-DNP N/ml serum	ABC·33 estimates (maximum percent antigen bound 20 ng DNP_7HSA added)	
			Serum dilution[a] 1:6	Serum dilution[b] 1:10
1 mg DNCB epicutaneous	1211	5.1	16	3
	1212	8.8	7	1
	1213	3.6	0	0
	1214	2.0	0	2
	1215	8.0	21	14
100 μg DNCB in CFA	1241	6.5	0	3
	1242	6.6	5	6
	1243	2.3	3	5
	1244	4.9	0	0
	1245	2.6	0	1

[a] Sera were further diluted serially to 1:96.
[b] Sera were further diluted serially to 1:160.

TABLE III. COMPARATIVE ESTIMATES OF ANTIGEN-BINDING CAPACITY BY THE mABC AND FARR ASSAYS.

Immunogen	Day bleeding	Guinea pig number	mABC assays (μg anti-DNP N/ml)	ABC-33 assays[a] Maximum percent antigen bound	
				20 ng DNP_7HSA added	2 ng DNP_7HSA added
1 mg DNCB epicutaneous	14	1252	3.0	7	16
	14	1253	2.0	0	7
	14	1254	10.0	7	5
	70	1232	6.1	0	0
	70	1233	9.5	0	0
	70	1234	8.1	8	0
100 μg DNCB in CFA	14	1262	14.3	15	24
	14	1264	9.1	10	19
	14	1265	11.0	0	15
	70	1263	29.0	11	30
	70	1264	38.0	10	12
	70	1265	23.7	15	31

[a] These assays were performed with serum dilutions ranging from 1:10 to 1:80.

TABLE IV. COMPARATIVE mABC AND ABC-33 VALUES OF GUINEA PIG ANTISERA TO DNP·BGG.

Immunogen	Expt. no.	No. of sera	mABC assays (μg antibody N/ml serum)		ABC-33 assays with μg AgN			
					0.2 (μg AgN bound/ml serum)		0.02	
			$\bar{m}$	Range	$\bar{m}$	Range	$\bar{m}$	Range
$DNP_{31}BGG$ 100 μg in CFA	F	9	175	89–234	24	9–39	9	2–21
	G	5	126	76–176	17	9–24	11	5–16
	H	10	94	50–135	11	7–17	3	2–8
	I	5	51	38–68	8	5–13	5	1–10

antibody content obtained after immunization with DNCB (13). This limitation was, however, overcome in Ouchterlony double-diffusion studies with appropriately adjusted antigen levels as shown in Fig. 2, which also attest to the specificity of the anti-DNP immunoglobulins. Further, the precipitation lines shown in Fig. 2 were not apparent when considerably more antigen, $DNP_{37}HSA$, was placed in the central wells. Crowle has cited other instances in which gel-diffusion analyses revealed precipitins that were not detectable in fluid media (14).

A comparison of the results obtained with the ABC-33 and mABC assays is of particular interest. Not a single serum of the 22 specimens examined by the Farr assay yielded unequivocally positive results. In striking contrast, application of the mABC procedure enabled the detection and estimation of antibody to DNP in weight units of 110 anti-DNCB sera which included the 22 mentioned above (1). The discrepancy arises from the following relatively simple considerations. The Farr-type assay is based on the use of a series of serum dilutions which are incubated with a constant but minimal amount of antigen in the submicrogram range. These conditions militate against the detection of antibody in sera of relatively low potency, a difficulty compounded when the immunoglobulins are of low avidity. The mABC procedure was designed to circumvent these restrictions through the use of antigen in a sufficiently large molar excess to register all immunoglobulins in complexes of the form Ag_2Ab. This simple modification diminishes the effect of dissociation and yields results which can be expressed in antibody weight units. As shown in Fig. 1 some sera such as No. 122 (7-day) fail to detect specific antigen binding even with an input of 20 μg antigen N. There is, therefore, little reason to suppose that positive results can be obtained with such sera in ABC-33 assays with antigen levels that are reduced by several orders of magnitude. The validity of the mABC assays is attested by the data in Table I. These data establish the close concordance of the results obtained with the mABC and quantitative precipitin analyses.

Summary. Antibodies in the sera of guinea pigs immunized with DNCB were detected by Ouchterlony-type analyses and were estimated in weight units by the mABC procedure. Assays of these sera by means of passive cutaneous anaphylaxis, passive hemolysis, or the ABC-33 antigen-binding technique were essentially negative. The validity of the mABC analyses was established by comparison of results obtained in quantitative precipitation studies with sera of guinea pigs immunized with DNP·BGG. In these instances, also, the ABC-33 assays failed to detect all the anti-DNP immunoglobulins.

1. Hurtado, Inés de, and Osler, A. G., Proc. Soc. Exp. Biol. Med. **149,** 628 (1975).
2. Chase, M. W., J. Exp. Med. **86,** 489 (1947).
3. Gell, P. G. H. and Benacerraf, B., J. Exp. Med. **113,** 571 (1961).
4. Frey, J. R., De Weck, A. E., and Geleick, H., J. Invest. Dermatol. **42,** 41 (1974).
5. Godfrey, H. P., and Baer, H., J. Immunol. **106,** 1201 (1971).
6. Osler, A. G., *in* "Methods in Immunology and Immunochemistry" (C. A. Williams and M. W. Chase, eds.), Vol. III, p. 73. Academic Press, New York (1971).
7. Farr, R. S., *in* "Methods in Immunology and Immunochemistry" (C. A. Williams and M. W. Chase, eds.), Vol. III, p. 63. Academic Press, New York (1971).
8. Hurtado, Inés de, and Osler, A. G., Proc. Soc. Exp. Biol. Med. **149,** 633 (1975).
9. Ovary, Z., *in* "Immunological Methods" CIOMS Symposium, (J. F. Ackroyd, ed.), p. 258 Blackwell, Oxford (1964).
10. Bloch, K. J., Kourilsky, F. M., Ovary, Z., and Benacerraf, B., J. Exp. Med. **117,** 965 (1963).
11. Eisen, H. N., Carsten, M. E., and Belman, S., J. Immunol. **73,** 296 (1954).
12. Mulligan, J. J., Jr., Osler, A. G., and Rodriguez, E., J. Immunol. **96,** 324 (1966).
13. Maurer, P. H., *in* "Methods in Immunology and Immunochemistry" (C. A. Williams and M. W. Chase, eds.), Vol. III, p. 1. Academic Press, New York (1971).
14. Crowle, A. J., "Immunodiffusion," Academic Press, New York (1973).

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