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# A SENSITIVE HEMAGGLUTINATION ASSAY METHOD FOR DINITROPHENYL-SPECIFIC ANTIBODIES

## The Effect of Antibody Binding Affinity on Titers[1]

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A sensitive assay method for 2,4-dinitrophenyl (DNP) specific antibodies which can be performed with minute amounts of serum would be desirable for diverse immunologic studies. This paper describes the development of such a hemagglutination (HA) assay system utilizing red blood cells (RBC) coupled with DNP groups. This method was found capable of detecting as little as 0.005 to 0.010 $\mu$g/ml of rabbit anti-DNP antibody protein. The effect of antibody-binding affinity upon the sensitivity is also described.

### MATERIALS AND METHODS

*Haptens.* Dinitrofluorobenzene (DNFB) was purchased from Eastman Organic Chemicals Co., Rochester, New York. Dinitrophenyl-$\epsilon$-aminocaproate (DNP-EACA) was prepared and crystallized as described previously (1). Other chemicals were of reagent grade.

*Rabbit anti-DNP sera.* Eleven serum pools were used. Each pool consisted of immune sera from two to five rabbits. Nine pools were prepared against $DNP_{50}$-bovine $\gamma$-globulin and two against $DNP_{138}$-bovine fibrinogen. Primary immunizations were with 2.0 to 10 mg of antigen in complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan) injected subcutaneously. Booster injections of 10 mg antigen intravenously were given 40 to 80 days after primary immunization. Sera were harvested either 32 days after primary immunization, or 7 days after booster immunization. DNP-specific antibody concentrations were determined by quantitative precipitation assay using Eisen's spectroscopic assay method (2) correcting for the optical contribution of antigen. The precipitating antigen was $DNP_{66}$-$PLL_{999}5$ (3). Precipitates were dissolved in 0.1N NaOH, $\epsilon 1\% = 15.8$, $\lambda$max 290 m$\mu$. Duplicate assays were done; average deviation from the mean, $\pm$ 5%.

*Purified antibodies.*[3] Purified anti-DNP antibodies were prepared from rabbit anti-($DNP_{60}$-B$\gamma$G) sera by the method of Farah, Kern and Eisen (4). Antibodies were eluted from specific precipitates with $DNP_{130}$-bovine fibrinogen using 2,4-dinitrophenol, and purified by dialysis and passage through a Dowex I ion exchange column. Antibody preparations were assayed for total $\gamma$-globulin concentration by spectrophotometry and were at least 85% specifically precipitable by $DNP_{130}$-fibrinogen. Rabbits were immunized with 5 mg of $DNP_{60}$-bovine $\gamma$-globulin in complete Freund's adjuvant injected into the footpads. Antibody preparations 6, 10, 14 and 34 were from individual rabbits bled on day 13 (for nos. 6 and 14) or day 27 (for nos. 10 and 34). Antibody preparation X was from a pool derived from five rabbits bled on day 56.

*Binding affinity measurements.*[3] Average equilibrium association constants (*Ko*) for purified antibody preparations were calculated from the quenching of antibody fluorescence by binding to $\epsilon$-DNP-lysine in phosphate-buffered saline, pH 7 at 26°C using a thermostated Aminco-Bowman spectrophotofluorometer as described by Velick, Parker and Eisen (5). Titrations were carried out and *Ko* were calculated as described by Eisen and Siskind (6).

*Buffers.* 0.300 osmolar pH 8.0 phosphate: 0.10 M $K_2HPO_4$ was neutralized to pH 8.0 $\pm$ 0.1 with 0.30 M HCl. 0.300 osmolar, pH 9.5, borate-KCl: 5 mmoles of sodium borate ($Na_2B_4O_7$) was dissolved with prolonged stirring into 100 ml of

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0.075 M KCl, and the solution was adjusted to pH 9.5 ± 0.1 with 0.30 M KOH. 0.300 osmolar, pH 7.0 phosphate (*wash solution*): 0.10 M $K_2HPO_4$ was neutralized to pH 7.0 with 0.30 M HCl. Osmolar concentrations were confirmed by measurement in a Fiske osmometer, (Fiske Association, Inc., Bethel, Connecticut).

*Sensitization of RBC with DNFB.* O+ human blood was drawn into Alsever's solution and washed five times with saline. Only freshly drawn RBC were used for sensitization. For reaction with DNFB, 0.50 ml of packed RBC was mixed with 10 ml of reaction buffer (borate-KCl, or pH 8. phosphate), and DNFB dissolved in 0.10 ml of acetone was added dropwise to the gently stirred RBC suspension. The reaction mixture was then mixed slowly on a Roto-Rack, model 96 (Fisher Scientific Instrument Division, Pittsburgh, Pennsylvania) at 24°C ± 1° for 1 hr. The sensitized RBC (DNP-RBC) were washed five times with pH 7.0 phosphate buffer by centrifugation. The washed cells were diluted to a 1% suspension in pH 7.0 phosphate buffer and used for hemagglutination. DNP-RBC were always used for HA on the day that they were prepared. After storage for 24 hr at 4° in a 0.30 osmolar pH 7.0 phosphate-glucose solution, their sensitivity decreased by one tube.

*Hemagglutination (HA) methods.* Serum diluents: The dextran-fetal calf serum-phosphate (FCS) diluent (dex-FCS-phos) was prepared by mixing two volumes of pH 7.0 phosphate buffer, one volume of 6% dextran in saline, and sufficient FCS to make a final concentration of 1:75 FCS. The 6% dextran solution was obtained as Gentran (Baxter Laboratories, Morton Grove, Illinois); average molecular weight of dextran, 75,000. Pooled FCS was purchased from Baltimore Biological Laboratory, Baltimore, Maryland. FCS was inactivated at 57° for 30 min, and absorbed once with DNP-RBC (1 vol DNP-RBC to 4 vol FCS) at 4°C for 3 hr.

HA titrations: The methods used in this study for DNP-specific HA are similar to those described in detail previously (7, 8) for benzylpenicilloyl (BPO)-specific HA. Briefly, sera were diluted initially in pH 7.5 phosphate-buffered saline, using different pipettes for each dilution step. Then 0.10 ml of the last dilution was mixed with 0.30 ml of dex-FCS-phos, and serial twofold dilutions were carried out by repeated transfers and mixing of 0.20 ml of the previous dilution with 0.20 ml of diluent. No more than five serial dilutions should be carried out to reach the end point, in order to avoid carry-over dilution errors. The serum dilutions were then mixed with 0.05 ml of the 1% suspension of DNP-RBC in homogeneous HA tubes. The mixtures were incubated at 25° for 45 to 60 min, shaken to resuspend the cells, and centrifuged for 1 min at 5°, 1000 rpm (head no. 269, International Centrifuge, Needham Heights, Massachusetts). Tap patterns were read. The titer was the highest dilution still showing weak but definite agglutination (see references 7 and 8 for detailed description of the method). DNP-RBC prepared in borate-KCl always gave negative diluent controls. DNP-specificity of HA reactions was always confirmed by specific hapten inhibition; after HA reactions were read, 0.05 ml of a $3 \times 10^{-3}$M solution of DNP-EACA in dex-FCS-phos was added to the serum dilution giving a 2+ to 3+ positive HA test, the tube was shaken for 5 sec on a Cyclo-Mixer (Clay Adams, Inc., New York, New York), and the HA pattern was reread. DNP-specific HA reactions became completely negative. DNP specificity of this inhibition was confirmed by two controls: 1) benzylpenicilloyl-EACA did not inhibit weak DNP-specific HA reactions when carried out as described above; and 2) DNP-EACA did not inhibit weak benzylpenicilloyl-specific HA reactions, or HA in the human ABO system, when carried out as described above.

## RESULTS

*DNFB-RBC coupling conditions and sensitivity of HA.* Two buffer systems were used for reaction of RBC with DNFB, phosphate buffer at pH 8.0 and borate buffer at pH 9.5. Reactions were run with different quantities of DNFB, at 24°C ± 1° for 1 hr, with constant mixing. Under these conditions reactions appeared to have gone to completion except in the cases where 8 and 16 mg of DNFB was used (Table I). The pH dropped a maximum of 0.2 units during the reactions. DNP-RBC were washed thoroughly and used to assay two standard rabbit anti-DNP sera. Table I shows that equal sensitivity of DNP-RBC was obtained with both buffer systems. The borate-KCl buffer was chosen as standard since DNP-RBC prepared in borate-KCl gave negative diluent controls, whereas DNP-RBC prepared in phosphate gave trace reactions with diluent alone.

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TABLE I

*Effect of dinitrofluorobenzene (DNFB) concentration on sensitization of red blood cells (RBC)*

| DNFB | Hemagglutination (HA) Titers[a] | | DNFB | Hemagglutination (HA) Titers[a] | |
|---|---|---|---|---|---|
| | Reaction buffers | | | | |
| | Borate-KCl pH 9.5 | | | Phosphate pH 8.0 | |
| | Test Sera 1B | Test Sera VIII | | Test Sera 1B | Test Sera VIII |
| *mg*[b] | | | *mg*[b] | | |
| 0.13 | 8000 | 20000 | 0.25 | 2000 | 10000 |
| 0.25 | 16000 | 80000 | 0.50 | 8000 | 20000 |
| 0.50 | 32000 | 160000 | 1.0 | 16000 | 80000 |
| 0.70 | 32000 | 160000 | 2.0 | 32000 | 160000 |
| 1.0 | 32000 | 160000 | 4.0 | 16000 | 80000 |
| 1.5 | 16000 | 80000 | 8.0 | 8000 | 40000 |
| 2.0 | Hemolysed | | 16.0 | 8000 | 40000 |
| 3.0 | Hemolysed | | | | |

[a] Averages of triplicate experiments; average deviation from the mean titer is less than one tube. Diluent: dex-FCS-phos. HA was specifically inhibited by dinitrophenyl-ε-aminocaproate.

[b] Per 10 ml buffer + 0.5 ml of packed RBC.

TABLE II

*Effect of serum diluent on hemagglutination (HA) titers*

| Standard Sera | Anti-DNP Antibody Protein Concentration[a] | HA Titers[b] | | | |
|---|---|---|---|---|---|
| | | Serum diluents[c] | | | |
| | | Phos | FCS-Phos | Dex-Phos | Dex-FCS-Phos |
| | *μg/ml* | | | | |
| 3B | 100 | 2000 | 2000 | 2000 | 16000 |
| 24A | 290 | 8000 | 8000 | 4000 | 64000 |
| VIII | 1100 | 4000 | 8000 | 4000 | 160000 |
| P | 3000 | 8000 | 16000 | 4000 | 320000 |

[a] By quantitative precipitin analysis.

[b] Averages of triplicate experiments; average deviation from mean titer is less than one tube. HA was specifically inhibited by dinitrophenyl-ε-aminocaproate at $6 \times 10^{-4}$M final concentration.

[c] Phos = 0.300 osmolar phosphate buffer, pH 7.0; dex = 2% dextran, av. molecular weight, 75,000; FCS = absorbed fetal calf serum, final concentration, 1:75.

In both buffer systems, sensitivity of the DNP-RBC first increased with DNFB concentration, and then decreased. There was only a trace of hemolysis throughout the DNFB quantity range in the phosphate system, and up to the 1.5 mg DNFB quantity in the borate system. Thus differences in hemolysis do not account for the decreased sensitization of RBC in the high DNFB concentration range. The 0.70 mg DNFB borate-KCl buffer reaction mixture (Table I) was chosen as optimal and was used for the remainder of this study.

*Effect of multiple washes on DNP-RBC sensitivity.* DNP-RBC were washed 5 times and 20 times and used to assay two standard rabbit anti-DNP sera. The sensitivity of the 20-times washed DNP-RBC was not reduced; HA titers were the same for 5-times and 20-times washed DNP-RBC.

*Effect of diluent on HA titers.* Table II shows that the use of a serum diluent containing fetal calf serum and dextran increased the HA titers 20- to 40-fold over HA titers of sera diluted in phosphate buffer. Both dextran and FCS must be present in the diluent to obtain optimal sensitivity. With this diluent, HA titers were reasonably proportional to precipitin titers (Tables II and III). HA was DNP-specific as indicated by specific inhibition with DNP-EACA at $6 \times 10^{-4}$M final concentration (see Methods).

*Sensitivity of HA system.* DNP-RBC were used to assay 11 different rabbit anti-DNP serum pools which contained from 61 to 3000 μg/ml anti-DNP antibody protein, as measured by quantitative precipitin analysis. The sera were obtained at least 30 days after primary immunization. Ten pools were obtained after booster injections. HA assays were done three times with different DNP-RBC preparations. Table III shows that HA titers of the 11 sera were reasonably proportional to their antibody concentrations. Sensitivity varied from 0.005 to 0.010 μg/ml of antibody protein. HA titers were reproducible; mean deviation from the average of triplicate assays was less than one tube. HA reactions were specifically inhibited by DNP-EACA as described in the Methods section.

*Effect of binding affinity of HA sensitivity.* Five samples of purified rabbit anti-DNP antibodies with known average binding affinities (*Ko*) were compared with regard to the antibody concentrations required to give threshold HA reactions. Table IV shows that the two relatively low-affinity antibody preparations required about a threefold higher antibody concentration (av. = 0.042 μg/ml) to elicit end point HA reactions than

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TABLE III

*Sensitivity of hemagglutination (HA) method*

| Rabbit Anti-Dinitrophenyl (DNP) Sera | Anti-DNP Antibody Protein Concentration[a] | HA Titers | | | | Anti-DNP Antibody Protein Concentration in End Point Tube |
|---|---|---|---|---|---|---|
| | | Triplicate tests[b] | | | Averages | |
| | *μg/ml* | | | | | *μg/ml* |
| 1 b | 300 | 32000 | 32000 | 32000 | 32000 | 0.0094 |
| VIII | 1100 | 160000 | 160000 | 160000 | 160000 | 0.0069 |
| 3 b | 100 | 8000 | 8000 | 16000 | 11000 | 0.0091 |
| 35 a | 630 | 80000 | 80000 | 80000 | 80000 | 0.0079 |
| 35 b | 265 | 40000 | 4000 | 40000 | 40000 | 0.0066 |
| 35 c | 200 | 32000 | 32000 | 64000 | 43000 | 0.0047 |
| 24 a | 290 | 64000 | 64000 | 64000 | 64000 | 0.0045 |
| 37 a | 61 | 4000 | 16000 | 8000 | 9000 | 0.0068 |
| 37 b | 185 | 16000 | 6200 | 64000 | 40000 | 0.0046 |
| P | 3000 | 200000 | 320000 | 400000 | 300000 | 0.0010 |
| II | 400 | 32000 | 64000 | | 50000 | 0.0080 |

[a] By quantitative precipitin analysis.

[b] Done with different dinitrophenyl-red blood cell preparations. HA was specifically inhibited with dinitrophenyl-ε-aminocaproate at $6 \times 10^{-4}$ M final concentration.

TABLE IV

*Effect of antibody-binding affinity on sensitivity of hemagglutination (HA) method*

| Antibody (Ab) Preparation | Anti-DNP Antibody Protein Concentration | Known Average Binding Affinities[a] | HA Titers | | | Anti-DNP Antibody Protein Concentration in End Point Tubes |
|---|---|---|---|---|---|---|
| | | | Duplicate tests | | Averages | |
| | *μg/ml* | *L/mole* | | | | *μg/ml* |
| 6 | 2620 | $3.5 \times 10^6$ | 80000 | 80000 | 80000 | 0.033 |
| 14 | 765 | $2.5 \times 10^6$ | 20000 | 10000 | 15000 | 0.051 |
| 10 | 2240 | $10^{10}$ | 320000 | 160000 | 240000 | 0.0093 |
| 34 | 356 | $10^9$ | 20000 | 20000 | 20000 | 0.018 |
| X | 3240 | $10^{11}$ | 320000 | 320000 | 320000 | 0.010 |

[a] See Methods for preparations and procedures.

did the three high affinity antibody preparations (av. = 0.012 μg/ml).

## DISCUSSION

The hemagglutination (HA) assay method for DNP-specific antibodies described above is sensitive, specific, reproducible and convenient. The principles governing the development of this method are discussed below.

Sensitization of RBC with DNFB appears to involve the chemical reaction of DNFB with RBC surface constituents to yield covalently bound DNP haptenic groups. This view is based on the known chemical reactivity of DNFB with primary amines and mercaptans under the conditions employed (9), and on the observation that repeated (20 times) washes of DNP-RBC did not decrease their specific agglutinability. Proof of this view will require degradative studies on "ghosts" of DNP-RBC.

Sensitivity of the assay method for 11 rabbit anti-DNP sera ranged from 0.005 to 0.010 μg/ml antibody protein, about 10- to 20-fold more sensitive than the DNP-specific HA assay method reported previously (10). The sensitivity of the present method is about the same as the sensitivity of HA assay systems employing protein antigens adsorbed onto tanned RBC or coupled by bis-diazobenzidine (11, 12). It is about 10- to 20-fold more sensitive than passive cutaneous anaphylaxis in the guinea pig (13), but about 10-fold less sensitive than the benzylpenicilloyl-specific HA assay system described previously (8).

This order of sensitivity was obtained by using "optimally sensitized" DNP-RBC and a serum

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diluent containing both dextran and fetal calf serum. As to "optimally sensitized" DNP-RBC, it is known that specific hemagglutination results from lattice formation between multivalent RBC and bivalent (or multivalent) antibodies (14–18) Thus there should be an optimal numerical range of hapten groups per RBC which would permit the formation of large or stable lattices at low antibody concentrations, i.e., in the end point tube. Our data are consistent with this view as sensitivity of DNP-RBC first increased then decreased with conditions which would be expected to couple increasing numbers of DNP groups onto RBC. However, these findings might also be due to nonspecific factors, such as charge or configuration of the RBC membrane, which would also effect the stability of antibody-RBC lattices. In either case, it is clear that "optimal sensitization" of DNP-RBC is necessary to obtain maximal HA titers.

The molecular mechanisms whereby the dex-FCS-phos diluent greatly increases HA titers are not known. Apparently, the effect of this diluent is to permit the formation of more stable antibody-RBC lattices at low antibody concentrations, either by increasing nonspecific attractive forces between RBC, or by decreasing the electrostatic repulsive forces generated during hemagglutination. With this diluent, HA titers of 11 rabbit anti-DNP sera reasonably paralleled their antibody concentrations, and could be used as a measure of antibody concentrations.

It was found that the sensitivity of the HA method was influenced by antibody-binding affinity; sensitivity was greater for the antibodies with relatively high affinity. However, in these experiments, the effect of affinity was relatively small. Sensitivity was increased only one to two tubes (2- to 4-fold) for a 10,000-fold increase in *Ko* ($10^6$ to $10^{10}$ liters per mol). This affinity effect can be accounted for on the basis that antibodies with relatively high affinity would be more likely to form stable antibody-RBC lattices at low antibody concentrations, i.e., in the end point tube. This affinity effect, although relatively small here, might be more striking if studied in a more sensitive assay system, e.g., one where the end point occurs at 100- or 1000-fold lower antibody concentrations, or where antibodies of considerably lower affinities are used.

This DNP-antibody assay method will be useful in situations where low antibody concentrations must be measured, and where only minute volumes of fluid are available for analysis. DNP-RBC may also be used to study single immunocompetent cells producing anti-DNP antibodies. The principles described herein have permitted the development of sensitive HA assay methods for antibodies of DNP and benzylpenicilloyl (7, 8) haptenic specificities, and may also be applicable to other haptens.

SUMMARY

A sensitive and specific passive hemagglutination (HA) assay for 2,4-dinitrophenyl (DNP)-specific antibodies is described. Red blood cells (RBC) are sensitized by coupling DNP groups to their surfaces. The sensitivity of the method was obtained by the use of "optimally sensitized" RBC and a serum diluent containing dextran and fetal calf serum. The method detects as little as 0.005 to 0.010 $\mu$g/ml of anti-DNP antibody protein. The method was found to be about three times more sensitive for antibodies with relatively high affinity (*Ko*, $10^{10}$ L/mole), than for those with low affinity (*Ko*, $10^6$ L/mole).

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