Antitrinitrophenyl (TNP) Plaque Assay. Primary Response of Balb/c Mice to Soluble and Particulate Immunogen[1] (34264)

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The hemolytic plaque assay introduced by Jerne and Nordin (1) and by Ingraham (2) permits localization and quantitation of specific cells producing antisheep red blood cell antibodies. Merchant and Hraba (3) modified the plaque technique to detect antibodies produced by rabbit spleen cells against the arsanilate hapten. Other investigators reported plaque assays to detect mouse cells producing antibodies against dinitrophenyl (4) and penicillin (5) haptens. Assays of cells producing antibodies against polysaccharides (6), synthetic polypeptides (7), and proteins (8–10) have been reported also. With the exception of the polysaccharide system, the cellular responses reported were small. Mice immunized against dinitrophenyl and penicillin were reported to average approximately 20 antihapten plaque forming cells (PFC) per million spleen cells after primary immunization (4, 5). It was not clear whether the methods were insensitive to many cells forming hapten-specific antibody or whether immunization failed to induce vigorous antihapten responses in mice. It was suggested that the multiple antigens of the erythrocyte stimulate a broader spectrum and, therefore, a larger fraction of the immunocompentent population than does the limited specificity of the haptenic determinant (4).

The research potential of a vigorous antihapten response in mice detectable by cellular methods made it desirable to determine whether the limitations of the antihapten plaque responses studied in mice previously were technical or biological. The hapten–protein conjugate, trinitrophenyl–hemocyanin (TNP-KLH) was shown previously to be a potent immunogen in rabbits (11). Precipitating antibodies were detected to the haptenic determinant after a single intravenous injection. We attempted to use the TNP-KLH to immunize mice and to develop a hemolytic plaque assay to TNP with Balb/c mice. The present study shows that immunization with TNP-KLH results in an antihapten response detectable by the plaque technique. The reagent used for assay, sheep erythrocytes coupled covalently to TNP, is stable for several days and yields both direct and indirect plaques (12, 13) in appropriately immunized animals. Immunogenicity of the hapten based on the number of plaque forming cells (PFC) elicited is more a matter of the form of the immunogen than the variety of obvious determinant groups used for immunization. Although soluble TNP-KLH induces an antihapten response in Balb/c mice, the response is greater when the immunogen is particulate.

Materials and Methods. Hapten reagent. Picryl sulfonic acid, 2,4,6-trinitrobenzene sulfonic acid, (TNBS) was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio and recrystallized from 1 *N* HCl (11).

Buffers. Cacodylate buffer, 0.28 *M*, pH 6.9 (11). Modified barbital buffer (*MBB*), pH 7.3–7.4 (14).

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Sheep red blood cells (SRBC). Normal SRBC were obtained at weekly intervals from Sheep Blood Supply, Tualatin, Oregon. Cells were aged at least 2 weeks after bleeding before use. Cells were washed three times in cold MBB. Suspensions which still showed hemolysis on the third washing were discarded and erythrocytes from a different bleeding were used.

Preparation of hapten coupled sheep red blood cells (HSRBC). A 60-mg portion of TNBS was dissolved in 21.0 ml of cacodylate buffer in a 50-ml Erlenmeyer flask. Three ml of packed SRBC were added dropwise with stirring. The mixture was stirred slowly with a magnetic bar at 23–25° for 10 min. Reacted cells were poured into a 50-ml conical glass tube which was then filled with cold MBB and mixed gently with a stirring rod. Cells were centrifuged for 5 min at 1230*g*. The supernate was decanted and 35 ml of cold MBB containing 0.022 g of glycyl–glycine were added (15). The mixture was stirred, centrifuged and decanted as above. The supernate containing glycyl–glycine–TNP was very yellow. A third washing in MBB as above was generally colorless or only very faintly yellowish-red. Subsequent washes should be colorless if the cells are stable. Washed HSRBC were stored with 30–40 ml of cold MBB in the refrigerator if not to be used immediately. Cells are stable for 3 or 4 days, but need to be washed 2–3 times before use. The haptenation reaction and subsequent handling of cells was carried out in foil-wrapped containers to protect against photodecomposition (14).

Animals. Balb/c female mice 3–5 months old were employed. They were caged in groups of 8 with free access to food and water. The mice were obtained from Simonsen Laboratories, Gilroy, California.

Immunogens. The TNP-KLH was prepared from keyhole limpet hemocyanin and TNBS (11). Mole ratios of hapten to carrier as described (11) ranged from 850–1000 moles of TNP/mole of KLH of assumed molecular weight 8×10^6.

The TNP-KLH was made particulate by coating it onto bentonite (Fisher Scientific) as Gallily and Garvey did with native KLH (17). Their method was modified in that the bentonite sediment after centrifugation of 40 ml of stock solution was resuspended in 2 ml of 0.28 *M* cacodylate buffer, pH 6.9, containing 5 mg of TNP-KLH/ml. The suspension was allowed to stand at room temperature for 1 hr with occasional shaking. After centrifugation particles were washed 3× in cacodylate saline (11). The amount of protein coated on the particles was determined by nesslerization (14).

Primary immunization consisted of a single injection of either soluble or particulate TNP-KLH. Secondary responses to particulate antigen were induced by two injections of TNP-KLH bentonite 14 days apart followed by a third, recall injection 1 month later. Each injection contained 100 μg of protein. All injections of either soluble or particulate immunogen were administered intraperitoneally.

Plaque assay. The method of Jerne *et al.* (18) was used. Guinea pig complement was purchased from Grand Island Biological Company, Grand Island, New York and used diluted 1:10 or 1:20. Mice were tested individually using 4 plates/spleen cell dilution. Data describing the secondary response were obtined after facilitation of 7S plaque formation with rabbit antimouse globulin serum (12, 13). Cells obtained from mice after secondary stimulation were washed once with 30 vol of Eagles medium (18) before plating The antiglobulin serum contained precipitating antibodies against mouse IgG and IgA but not IgM as determined by immunoelectrophoresis. This serum inhibited early primary responses approximately 25–30% probably because of an anti-IgM component too weak to detect by immunoelectrophoresis. The facilitation serum was absorbed 3× with SRBC (19). It was used at 1:100 dilution which was the dilution of maximum efficiency determined by titration. Facilitation serum was added to plates 90 min after initiation of incubation. The plates were incubated an additional 45 min, the antiglobulin was poured off, and complement was added. The plates were incubated an additional 45 min.

Plaques were counted on a New Brunswick Scientific colony counter with electronic

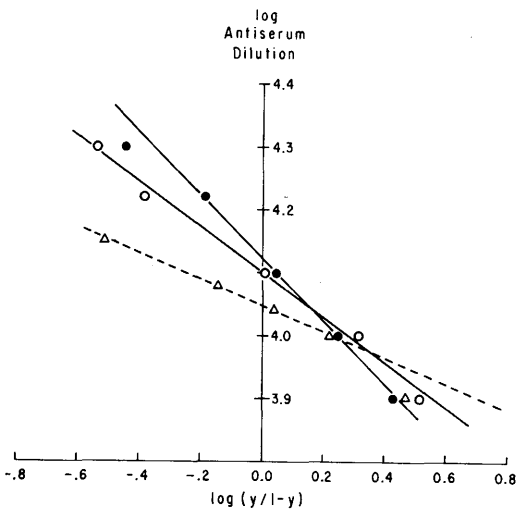

FIG. 1. Lysis of native (SRBC) and haptenated sheep RBC (HSRBC) by standard anti-TNP serum or rabbit antisheep RBC (hemolysin). Plotted according to Von Krogh as described in (14), where Y = degree of lysis expressed as a fraction of 1. The antilog of the intercept at the ordinate equals the 50% hemolytic unit. (△), lysis of HSRBC by anti-TNP; (●), lysis of HSRBC by hemolysin; (○) lysis of SRBC by hemolysin. Dilution of anti-TNP serum was multiplied by 10 to permit plotting on a common scale.

probe. The plates were stained with benzidine before counting (18).

Hapten Inhibition. The TNP–bovine serum albumin (TNP-BSA) was synthesized as in (20). It contained 14 moles of TNP/mole of BSA, molecular weight 70,000. For inhibition, 0.1 mg/ml of TNP-BSA was incorporated into the soft agar prior to plating.

Hemolytic titration. Immune hemolysis of native and haptenated erythrocytes in MBB was as described in (15). The method was modified in that the lytic reaction was stopped by adding 0.9 ml of cold isotonic citrate–saline prior to spectrophotometric measurement (19). Antisheep cell hemolysin was purchased from Grand Island Biological Company. Rabbit anti-TNP antiserum 8-57 was described previously (20). It contained 806 μg of anti-TNP/ml. The anti-TNP serum was absorbed 3× with SRBC (19). Hemolysis was determined in a Beckman DBG spectrophotometer at 530 mμ; 50% hemolytic units were determined graphically. Log serum dilution was plotted against log $[y/(1 - y)]$ as in Fig. 1 where y is the degree of lysis expressed as a fraction of 1 (14). Samples were tested in duplicate and the values were averaged.

Results. Successful haptenation of SRBC by TNBS was judged initially by passive hemolysis (21) with a standard rabbit anti-TNP antiserum. Fig. 1 illustrates typical results. The anti-TNP serum was absorbed with normal sheep cells and did not lyse SRBC control cells tested simultaneously. Lysis of HSRBC by anti-TNP shown in Fig. 1 by the broken line indicates the presence of hapten on the red cell. According to these results the antihapten serum contained one thousand one hundred twenty three 50% hemolytic units/ml. The average of five such determinations was 1198 units/ml. Based on the anti-TNP precipitin content of the serum (806 μg/ml) each 0.11 μg of Ab N constituted one 50% hemolytic unit. This is similar to the value obtained by Rangel and Repka (22) for the 50% hemolytic unit in passive lysis measurements of anti-protein antibodies.

Solid lines in Fig. 1 represent lysis by rabbit anti-SRBC. Both haptenated and native RBC were lysed. The 50% hemolytic unit was not altered greatly when hemolysin reacted with HSRBC compared to SRBC. However, the degree of lysis was not identical at all antiserum concentrations. Comparisons of conditions of haptenation were made by the hemolytic method and it was on this basis that the described haptenation procedure was developed.

Primary immunogenicity of soluble TNP-KLH was estimated by injecting varying doses ranging by log increments from 10^{-4} mg to 1.0 mg/mouse. Comparison of responses of 41 immunized animals 5–7 days after injection indicated that 0.1 and 1.0 mg were the most immunogenic doses and yielded similar results. The mean response of 12 mice injected with 0.1 mg of TNP-KLH was 47 ± 12 anti-TNP PFC/10^6 spleen cells where ± 12 represents the 95% confidence limit of the mean. Of the 41 immunized mice, none contained more than 90 anti-TNP PFC/10^6 spleen cells or less than 15/10^6. In contrast, of 12 animals injected with diluent, the aver-

age anti-TNP PFC was 2.5 ± 1.0/10^6 spleen cells with no animal containing more than 6 PFC/10^6. The plaques formed were complement dependent and were inhibited by TNP-BSA. Each mouse immunized with soluble TNP-KLH was tested simultaneously against

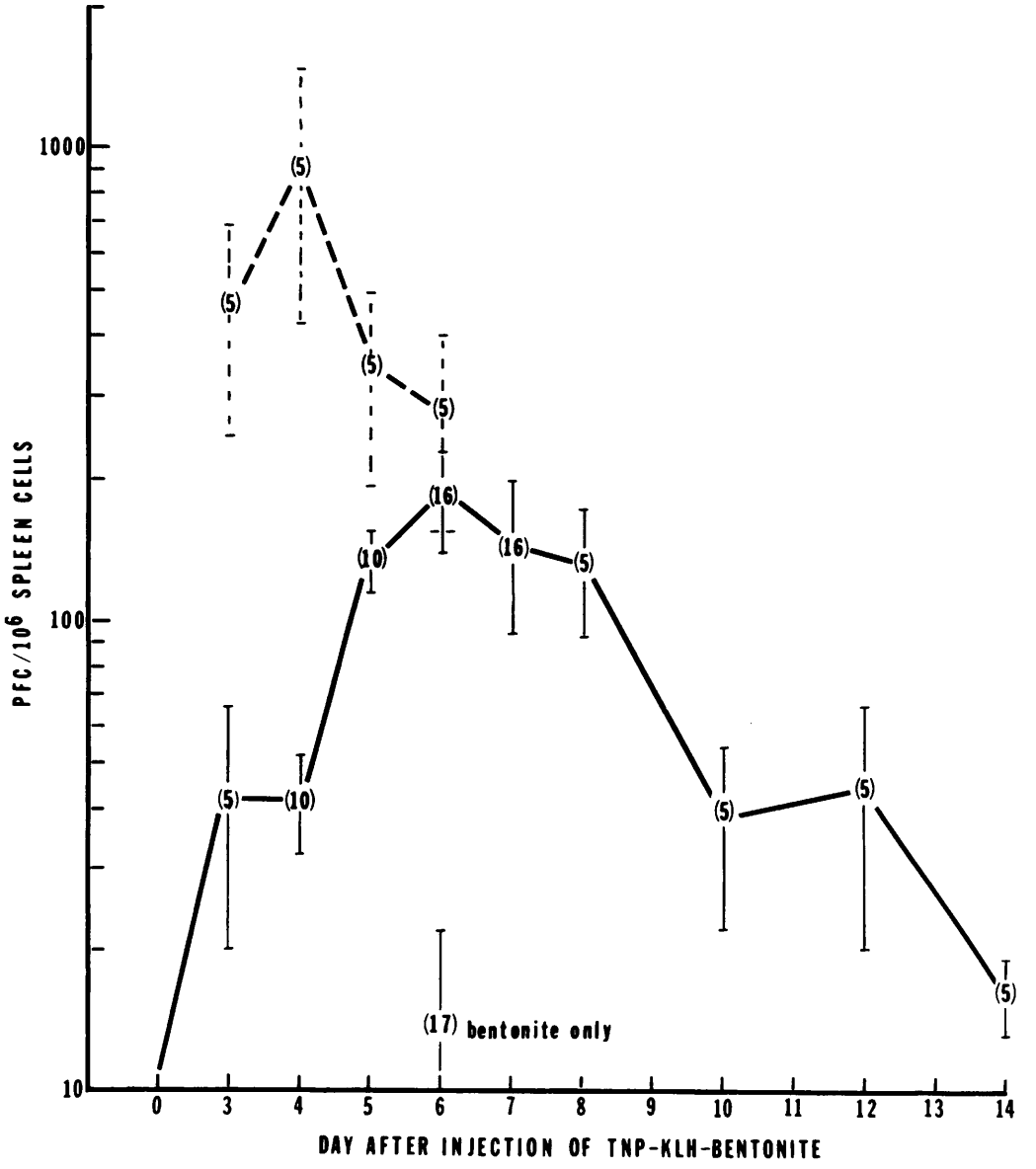

FIG. 2. Primary and secondary response to TNP-KLH–bentonite as detected by hemolytic plaque assay in gel using TNP-sheep RBC as indicator. Semilog plot of number of plaque forming cells (PFC)/10^6 spleen cells plated. Numbers in parentheses indicate number of individual mice tested per point. The parenthesis is located at the mean with the vertical bars indicating the 95% confidence limit. Primary mice received 100 μg T-K-B intraperitoneally on day 0. Secondary mice received two injections of 100 μg of T-K-B 14 days apart followed by challenge 1 month later. The secondary response is given in days after last injection. Day 0 level of $<$ 10 PFC/10^6 represents 13 mice injected with cacodylate–saline (antigen diluent) and tested 5–7 days later. Average anti-TNP PFC/10^6 = 2.5 ± 1 on day 0. Solid line = primary. Broken line = secondary.

HSRBC and SRBC. For all immunized mice the average plaque count against SRBC was 1.0 ± 0.8 PFC/10^6 spleen cells. Although primary immunogenicity was established for soluble TNP-KLH, the results were not particularly encouraging with respect to its practical use. Consequently, experiments were conducted with the particulate immunogen TNP-KLH–bentonite (T-K-B).

Preliminary experiments indicated that as with soluble TNP-KLH 100 μg T-K-B was more immunogenic than lower doses. For comparison, therefore, subsequent tests were conducted with 100 μg. Figure 2 shows primary and secondary antihapten responses of Balb/c mice injected intraperitoneally with T-K-B. The peak of the primary response to TNP appears between days 5–8. Thus the antihapten primary response to 100 μg of TNP-KLH is somewhat slower in developing than is the primary response to 10^9 sheep erythrocytes which generally peaks at day 4–5 (24). On the sixth day no animal had less than 50 anti-TNP PFC/10^6 spleen cells and 6/16 mice contained > 200 PFC/10^6. One mouse spleen contained 412 PFC/10^6 spleen cells. The primary results shown were obtained by direct plating and, therefore, are due mainly to cells forming 19S antibody (12, 13). Facilitation of these cell suspensions with antiglobulin reagent increased the average PFC/10^6 approximately 2-fold on days 7 and 8 but less than that on days 6 and 10. Of 17 control mice injected with bentonite alone and sampled on the sixth day, the average anti-TNP response was 14 ± 8 PFC/10^6. This effect of bentonite was surprising and was associated only with HSRBC, the average with SRBC being 0.5 ± 0.3 PFC/10^6. Plaques formed as a result of bentonite injection were inhibited 36% by 0.1 mg/ml of TNP-BSA; whereas primary plaques formed to T-K-B were inhibited 64%. Analysis[3] of the bentonite preparation indicated that it contains approximately 0.13% carbon and no detectable nitrogen. The kind of structure formed by the carbon is under investigation.

Primary responses induced by 100 μg of T-K-B subsided quickly. By day 14 little anti-TNP activity remained detectable in spleen cells. Primary sensitization was confirmed by challenge with 100 μg of T-K-B a month after two sensitizing injections administered 2 weeks apart. The broken line in Fig. 2 indicates the result of anamestic stimulation which produced a prompt response peaking several days earlier than the primary. The data shown are results obtained by facilitation with antiglobulin serum. In contrast to the primary response in which most of the component apparently was 19S, the secondary response appeared to be almost entirely 7S. None of the 20 mice had more than 32 PFC/10^6 spleen cells by direct plaque assay. A comparable group was not tested prior to secondary challenge, however in other experiments primed mice tested 1 month after sensitization have always averaged less than 100 PFC/10^6 by either direct or facilitated plaque assay.

The specificity of the secondary anti-TNP response is shown in Table I in which 0.1 mg/ml of TNP-BSA was incorporated into the soft agar prior to plating. Anti-TNP plaques were completely inhibited by the hapten while cells from control animals immunized with SRBC were unaffected.

Discussion. Direct haptenation of sheep erythrocytes by treatment with TNBS has provided a sensitive reagent capable of detecting cells producing anti-TNP antibody by lysis in gel. TNP-SRBC are stable for several days and in fact have been mailed successfully for use in another laboratory. Lysis of TNP-RBC is brought about by anti-TNP antibody but not by anti-KLH antibody and is inhibited by TNP-BSA. Whereas primary anti-TNP plaques are inhibited approximately 60% by 0.1 mg/ml of TNP-BSA, secondary (facilitated with antiglobulin serum) plaques are totally inhibited. We interpret this to reflect the weaker affinity of anti-TNP released by primary immune cells as was shown by Steiner and Eisen (25) for antidinitrophenyl. It is possible also that the antiglobulin reagent "fixes" the antibody so that it does not readily dissociate from the hapten-carrier complex.

The increased antihapten background (14 PFC/10^6) by bentonite alone is unexplained.

[3] Analysis performed by Berkeley Analytical Laboratories, Berkeley, California.

TABLE I. Hapten[a] Inhibition of Secondary Anti-TNP Plaque Response.

Immunizing antigen[b]	Mouse no.	PFC/plate[c]	
		No hapten	Hapten added
TNP-KLH–bentonite	1	61, 70, 68, 79	0, 0, 1, 0
	2	87, 92, 96, 89	0, 1, 2, 1
	3	90, 85, 92, 76	0, 1, 0, 0
	4	21, 23, 24, 32	0, 0, 2, 0
	5	101, 94, 103, 102	4, 6, 3, 5
Sheep-RBC	1	327, 315, 347	333, 335, 347
	2	239, 237, 221	195, 162, 225
	3	102, 94, 101	129, 113, 154
	4	181, 251, 218	175, 175, 172

[a] TNP-BSA was included in diluent prior to plating; final concentration 0.1 mg/ml.

[b] Immunization as in Fig. 1 for TNP-KLH mice Nos. 1–4 tested on the sixth day after last injection, No. 5 on the seventh day. SRBC mice were injected twice with 0.5 ml of 20% SRBC 5 months apart and tested on the fourth day.

[c] T-K-B mice: (Nos. 1–4), 2×10^5 spleen cells plated; (No. 5), 4.8×10^5 spleen cells plated. Anti-SRBC mice 4.5×10^4 spleen cells plated. Plates were treated with antiglobulin serum. Counts shown are total counts after facilitation.

Elemental analysis has revealed the presence of carbon but not nitrogen in bentonite (aluminum silicate). Plaques induced by bentonite injection are approximately 30% inhibitable by TNP-BSA. In that sense they appear hapten-specific but of weak affinity if our interpretation of the difference in degree of inhibition by hapten of primary and secondary cells is correct. In preliminary experiments conducted thus far, it has not been possible to increase the number of anti-TNP plaques induced by bentonite by its repeated administration. Balb/c mice are highly susceptible to the induction of myeloma tumors. It has been reported that about 10% of 240 mouse myeloma proteins had antibody activity against nitrophenyl derivatives or other ring structures (26). Originally we thought the induction of anti-TNP antibody by bentonite could be related to this phenomenon. However, induction by bentonite is not restricted to Balb/c but has occurred in other strains to the same extent. Nonetheless there might be such an association. Investigation of the bentonite induced anti-TNP response is in progress.

Immunization was successful both for soluble and particulate TNP-KLH. This is in accord with the findings of Gallily and Garvey (17) who reported that native KLH made particulate by adsorption to bentonite was much more immunogenic than soluble KLH. They attributed this enhanced immunogenicity to greater efficiency of uptake of particulate KLH by macrophages. We have not attempted to trace the fate of T-K-B within organs but have noted small white nodules adjacent to the spleens of many bentonite- or T-K-B-injected mice. Histopathologic examination indicated they were granulomatous lesions resembling those described by Wilkinson and White (27) in bentonite-injected guinea pigs.

Successful immunization with T-K-B is not limited to Balb/c mice. Results comparable to Balb/c have been obtained also with C57BL, DBA/2, and RF mouse strains. A dose of 100 μg of T-K-B induces a satisfactory primary sensitization within 6 days after injection. This dose was selected for purposes of comparison with soluble TNP-KLH and is probably not optimal. We have evidence that larger doses induce greater primary responses on the sixth day. The primary anti-TNP response induced by T-K-B is not appreciably less than that detected after primary immunization with sheep RBC. Previously reported weak antihapten plaque responses in mice (4, 5) appear to have suffered more from technical failure either in detection or

immunization than from an inherently weaker immunogenic potential of haptens compared to erythrocytes because of the number of different determinant groups.

Summary. A technique was developed for the detection of individual cells producing anti-TNP antibody by the hemolytic plaque technique. Conjugation of TNP directly to the erythrocyte surface by use of TNBS resulted in a stable reagent that permitted a study of the antihapten response to TNP-KLH. It was possible to induce a primary anti-TNP response with soluble TNP-KLH but the response was greater when the immunogen was made particulate by coating it onto bentonite. Both primary and secondary responding cells (those brought out by anti-globulin serum) were inhibited by TNP-BSA added to the plating medium but at an equivalent concentration of hapten only the secondary cells were completely inhibited. This was interpreted to indicate the higher binding affinity of secondary antibody.

Author's note. As we were in process of submitting this manuscript, we became aware of an abstract which indicated that responses of similar magnitude to those reported here with TNP could be obtained in Balb/c mice to DNP using a DNP-BSA-SRBC plaquing reagent. [H. Yamada and A. Yamada, Federation Proc. **28**, 428, (1969)].

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