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Antibodies with Multiple Binding Functions. Induction of Single Immunoglobin Species by Structurally Dissimilar Haptens

(antibody heterogeneity/isoelectric focusing/combining region/cell-surface receptors)

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ABSTRACT **Anti-hapten sera prepared in rabbits contain individual immunoglobulin species capable of binding several pairs of structurally diverse haptens A and B. (A = inosine, uridine, menadione, vitamin K_1, ribonuclease; B = 2,4-dinitrophenyl). In antisera against hapten A subjected to isoelectric focusing, there are many anti-A immunoglobulin species, but only a small proportion of these bind both A and B. When rabbits are primed with haptens A coupled to a carrier and then challenged with hapten B–carrier complex, there is an early restricted response of those species that bind both A and B. Later, immunoglobulins appear which bind B, but not A. These results suggest that multiple-binding antibodies exist in antisera against hapten and that such multiple binding is functional; i.e., that when two diverse haptens A and B bind to an immunoglobulin, both haptens may stimulate the cell-surface receptor to induce production of this immunoglobulin. Such phenomena may also provide a molecular basis for maturation of the immune response.**

Immune sera characteristically show a high degree of specificity for the antigen used to elicit the immune response. It might be expected that the individual immunoglobulins that constitute the serum might also show a similar high degree of specificity. Talmage proposed a model whereby different immunoglobulins might each have a partial fit for antigen and in which the specificity of the immune serum was due to the sum of several different partial fits (1). The mouse immunoglobulin IgA_2 myeloma protein 460 studied by Eisen (2) binds competitively the dissimilar haptens ϵ-2,4-dinitrophenyl-L-lysine (Dnp Lys) and 2-methyl-1,4-napthoquinone thioglycollate (MenTG) to different subsites in the combining region (3). Since several workers have obtained evidence suggesting that individual elicited antibodies may bind several structurally diverse antigens (2–6), we examined individual antibodies from anti-hapten sera prepared in rabbits to see if they also bind more than one structurally diverse antigen. We further asked that when one immunoglobulin binds both antigens A and B, whether both A and B induce the immune response of the same immunoglobulin.

MATERIALS AND METHODS

New Zealand white male rabbits were immunized with 5 mg of hapten–protein complex in complete Freund's adjuvant, subdermally in alternate toe pads. The second immunization (boosting) was with 5 mg of hapten–protein complex injected in incomplete Freund's adjuvant in the remaining toe pads. Presence of antibodies was detected by two-dimensional microdiffusion (7).

Isoelectric focusing and radioautography was done as described (8, 9), except that the polyacrylamide gels contained 4 M deionized urea. Antibodies were partially purified by precipitation with sodium sulfate (final concentration, 180 g/liter) three times and were dissolved in phosphate-buffered saline [0.01 M Na-phosphate–0.15 M NaCl (pH 7.4)] containing 4 M urea.

Whole antibody populations were screened for cross-reactivity (Varga, J. M. & Richards, F. F., in preparation). Briefly, partially hydrolyzed nylon mesh discs were used as initiator sites for polymerization of serine-*N*-carboxyanhydride (Pilot Chemicals) to polyserine chains. Antibodies against Dnp were coupled to the hydrophilic polyserine chains with glutaraldehyde. Crossreaction of a hapten with the antibodies against Dnp was measured by its inhibition of [^{3}H]ϵDnp-lysine binding.

$Dnp_{60}BGG$ and $Tnp_{55}BGG$ (2,4,6-trinitrophenyl-bovine gammaglobulin) were prepared according to Little and Eisen (10). $U_{58}BGG$ ($uridine_{58}$ BGG) was prepared by the cyanogen bromide activation method (11), and purified on a 3 × 60-cm Sephadex G-200 column developed with phosphate-buffered saline. $U_{52}KLH$ was prepared by a similar method. $Men_{25-34}BGG$ was prepared from 2-methyl-3-amino-1,4-naphthoquinone. Menadione bisulfate trihydrate (10 mmol) was dissolved in 10 ml of acetic acid. Sodium azide (10 mmol) dissolved in 5.0 ml of distilled water was added drop by drop over 20 min with stirring; 2-methyl-3-amino-naphthoquinone was precipitated, washed, and recrystallized from hot water three times. Mass spectrometry gave an M^{-1} ion at m/e 190 and the correct elemental composition on high-resolution mass spectrometry. Titration showed the presence of a single free amino group. To 5.5 mmol of 2-methyl-3-amino-1,4 naphthoquinone in 5 ml of 0.1 M sodium phosphate (pH 6.8), 5.5 mmol of glutaraldehyde and then 50 μmol of BGG was added and stirred at 20° for 14 hr. After dialysis the dissolved and precipitated MenBGG (25–34 menadione residues/7S monomer BGG) were combined as a suspension. $Folic\ acid_{20}BGG$ was prepared according to Ricker and Stollar, and the hapten is attached to the protein presumably by both its carboxyl and amino groups (12).

[^{125}I]Tyrosyl-ϵ-2,4-dinitrophenyl-L-lysine·hydrochloride (TyrLysDnp) was synthesized by radioiodination (13) of tyrosyl-ϵ-2,4-dinitrophenyl-L-lysine·HCl (a gift from Dr. Peter Jackson). The specific radioactivity was 60 Ci/mmol.

Abbreviations: BGG, bovine gammaglobulin; Dnp, 2,4-dinitrophenyl; Tnp, 2,4,6-trinitrophenyl; [^{125}I]TyrLysDnp, [^{125}I]L-tyrosyl-ϵ-2,4-dinitrophenyl-L-lysine; KLH, keyhole limpet hemocyanin; Men, menadione (2-methyl-1,4-naphthoquinone); V_H and V_L, variable (N-terminal) region of immunoglobulin, heavy chain and light chain, respectively.

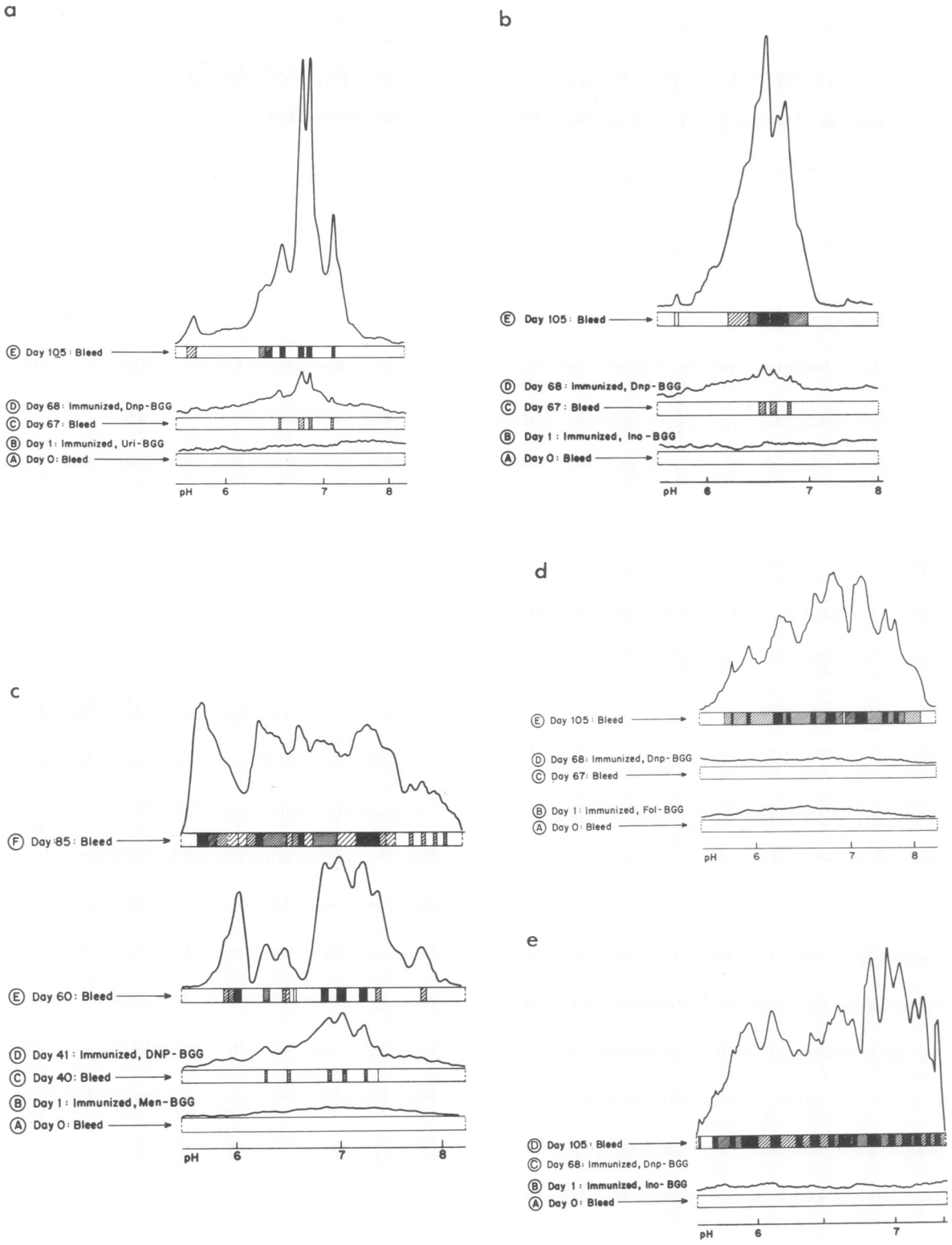

FIG. 1. Autoradiographs and densitometry tracings of the original autoradiographs (— —) of ampholyte gels containing rabbit immunoglobulins subjected to isoelectric focusing. The gels were treated with [^{125}I]TyrLysDnp to label Dnp-binding immunoglobulins (except in *e*). Intensity of shading represents the degree of blackness of the film. (*a*) Rabbit was immunized with uridine-BGG; and boosted with Dnp-BGG. (*b*) Rabbit was immunized with inosine-BGG and boosted with Dnp-BGG. (*c*) Rabbit was immunized with menadione-BGG and boosted with Dnp-BGG. (*d*) Rabbit was immunized with folic acid-BGG and boosted with Dnp-BGG. (*e*) The same serum as illustrated in gel *E* of *b*. The gel was incubated with inosine-[^{125}I]tyrosine and radioautographed. The day number of each bleeding refers to the days elapsed since the primary immunization.

N-iminoinosine-[3-^{125}I]-4-hydroxycinnaminic acid (inosine-[^{125}I]tyrosine adduct) was synthesized by adding 4.4 mM inosine to 4.0 mmol of cyanogen bromide; the pH was adjusted to 10.0–10.5, and the mixture was stirred for 5 min at 20°. The pH was adjusted to 8.5; 4.4 mmol of solid L-tyrosine were added and the mixture was stirred for 18 hr at 4°. A precipitate (unreacted L-tyrosine) was removed; the filtrate was adjusted to pH 2.0 with 1 N HCl and extracted three times with ethyl acetate; the aqueous phase was freeze-dried, dissolved in distilled water, and filtered. The solution was adjusted to pH 7.8 and stirred for 2 hr at 20°; the precipitate was collected. The precipitate was dissolved at pH 2.0, and the precipitation step at pH 7.8 was done twice more. The product had an $A_{248/280}$ nm ratio of 3.3 (ratio for inosine = 7.7; ratio for tyrosine = 0.15). The product contained 0.97 mol of tyrosine per mol. Mass spectrometry gave a large M^{-1} ion peak at m/e 420 and a small M^+ ion peak at m/e 421, indicating formation of an inosine–tyrosine adduct. This adduct was radioiodinated with ^{125}I (13); iodination time was 5.0 min. The product is probably an admixture of the mono and diiodo derivative. Specific radioactivity was 12 Ci/mmol.

RESULTS

Screening of Anti-Hapten Sera for Crossreactivity. Three rabbits were immunized with $Dnp_{60}BGG$ and boosted 1 month later with $Dnp_{60}BGG$. Antisera against Dnp were pooled. The partially purified anti-Dnp immunoglobulin population was screened for the ability to bind dissimilar haptens, by the nylon polyserine disc method outlined above. Of 144 haptens tested (36 amino acids and peptides, 17 carbohydrates, 14 vitamins and coenzymes, 14 proteins and antibiotics, 25 dyes, 37 nucleic acids, bases, and 21 other chemicals), five structurally diverse hapten pairs: Uridine-Dnp, inosine-Dnp, menadione-Dnp, ribonuclease-Dnp, and vitamin K_1-Dnp, crossreacted. Four of these are reported in detail. The fifth pair, vitamin K_1-Dnp, gave results closely resembling those for menadione–Dnp. Most pairs of haptens chosen at random do not crossreact. One such pair, folic acid-Dnp, is reported here in detail. We have confirmed that all our rabbits produced anti-folic acid immunoglobulins. Rabbit antiserum to ribonuclease does not crossreact with BGG.

Immunoglobulin Cross-Stimulation by Crossreactive Hapten Pairs. Rabbits were immunized with uridine-BGG and their sera were tested for the presence of antibodies against uridine at weekly intervals. When titers were between 200 and 1000 μg/ml of serum, the rabbits were bled at weekly intervals and the whole gammaglobulin fraction was isolated from the serum by precipitation with sodium sulfate and then subjected to isoelectric focusing. The gels were treated with [^{125}I]TyrLysDnp, washed, and radioautographed. 68 Days after primary immunization, there are four faint bands between pI 6.5 and 8.0 that bind the hapten [^{125}I]TyrLysDnp (Fig. 1*a*). These bands were abolished when the gammaglobulin fraction was pretreated with antibody against rabbit gammaglobulin prepared in goats, indicating that these bands were not due to protein contaminants such as albumen precipitated with the gammaglobulin fraction. Pretreatment of the rabbit gammaglobulin fraction with anti-rabbit Ig A globulin prepared in goats produced no visible change in the isoelectric focusing patterns. As IgM immunoglobulins do not enter the gel, we observe predominantly changes in the Ig G immunoglobulin pattern. In the presence of 0.1 mM uriding, all labeling with [^{125}I]TyrLysDnp was abolished, suggestine that immunoglobulin species binding competitively *both* uridine and [^{125}I]TyrLysDnp were present in each of these bands. Similar results were obtained with the other hapten pairs showing crossreactivity (Fig. 1*b* and *c*) and with RNase and Dnp (see Fig. 3).

On day 66, the rabbits immunized with uridine-BGG were further injected with 5 mg of $Dnp_{60}BGG$. Fig. 1*a* shows that there was a rapid early increase in those bands that bind both uridine and [^{125}I]TyrLysDnp and that these reached their maximum intensity at about 100 days. Fig. 2*A* is an autoradiograph of [^{125}I]TyrLysDnp-labeled gel on which the immunoglobulins of an early anti-uridine response have been resolved and which shows those bands that bind *both* uridine and TyrLysDnp. Such double-binding bands characterize the early response to boosting with Dnp-BGG and form a highly "restricted" immune response. Later, after the Dnp boost, bands that bind only TyrLysDnp appear, and there is a gradual increase of complexity in the anti-Dnp response (Fig. 2*B*) which eventually approaches the primary anti-Dnp response in complexity (Fig. 2*C*). Autoradiograph tracings were taken to measure the increase in the double-binding (uridine + TyrLysDnp) bands before and after boosting with Dnp-BGG. The areas under the curve of the densitometric tracing of double-binding bands increased 30- to 80-fold after boosting with the second hapten in the uridine-Dnp system (Fig. 1*a*). Similar results were observed with inosine-Dnp (Fig. 1*b*), menadione-Dnp (Fig. 1*c*), and ribonuclease-Dnp (Fig. 3). When the rabbits were not boosted, there was no substantial increase in the density of "double-binding" bands. Identity of the individual doubly reacting bands, before and after boosting, was established by cutting out individual bands before and after boosting, mixing the bands, and rerunning according to the method of Williamson (14). Double-binding bands, resolved at the same pI value, ran as a single band after mixing. Fig. 1*e* shows the appearance of gel E in Fig. 1*b* when reacted with inosine[^{125}I]tyrosine and radioautographed. This figure shows that the pattern of antibodies that bind A + B after boosting with B is not an artifact due to a general reduction in anti-A immunoglobulins.

The Antigen Pair, Ribonuclease and Dnp. Fig. 3 shows that when a rabbit is immunized with RNase, there is a relatively large number of partially resolved immunoglobulin bands that bind TyrLysDnp as well as RNase. Before boosting with Dnp-BGG (41 days after RNase immunization), TyrLysDnp binding to these bands is almost completely inhibited by pretreating the gel with 70 μM ribonuclease, indicating that all TyrLysDnp-binding bands also bind RNase competitively, and that hapten pairs as diverse as RNase and Dnp produce the same type of binding patterns as the hapten pairs uridine-Dnp, inosine-Dnp, and menadione-Dnp. When such RNase-primed animals are boosted with Dnp-BGG, two processes occur. The bands containing the double-reacting immunoglobulins (i.e., those that bind TyrLysDnp, but are inhibited by RNase) show the usual large increase in quantity on boosting with Dnp-BGG. These bands are centered around pI 6.8. A second effect after Dnp-BGG boosting is that new

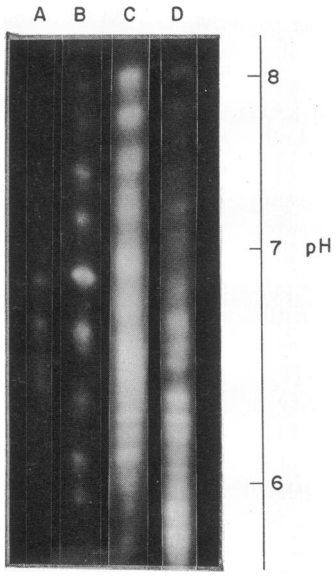

FIG. 2. Autoradiographs of ampholyte gels containing rabbit immunoglobulins subjected to isoelectric focusing. The gels were treated with [^{125}I]TyrLysDnp to label Dnp-binding immunoglobulins. Autoradiographs were recorded by contact photocopying; therefore, the photograph is a negative picture of the original autoradiograph. (*A*) Photographic appearance of an early "restricted" response consisting of antibodies that bind uridine + TyrLysDnp produced by priming a rabbit with uridine-BGG and boosting with Dnp-BGG. This pair of haptens cross-react. The serum sample was taken 25 days after Dnp-BGG boosting. (*B*) A later immune response in the same rabbit as (*A*) (46 days after Dnp-BGG boosting). Additional bands appear that bind TyrLysDnp, but in which the binding is not inhibited by uridine. The strong bands binding both uridine + TyrLys-Dnp are still present. The pattern approaches in complexity a primary anti-Dnp-BGG response. (*C*) A primary anti-Dnp-BGG response (25 days after immunization). (*D*) This rabbit was primed with folic acid-BGG and boosted with Dnp-BGG. The serum sample was taken 25 days after Dnp-BGG boosting. This pair of haptens does not crossreact. There is no "restricted" response and the isoelectric focusing pattern resembles in complexity that of a primary anti-Dnp-BGG response.

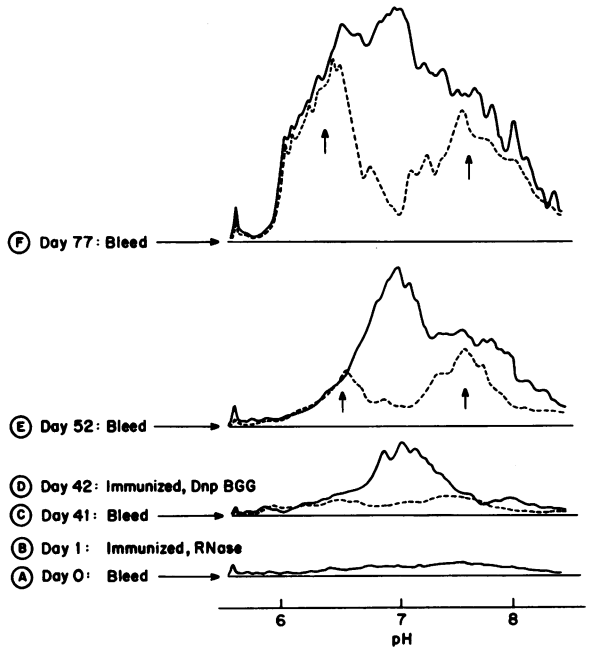

FIG. 3. Densitometry tracings of autoradiographs (——) of ampholyte gels containing rabbit immunoglobulins subjected to isoelectric focusing. The gels were treated with [^{125}I]TyrLysDnp to label Dnp-binding immunoglobulins. The rabbit in this experiment was immunized with RNase and boosted with Dnp-BGG. Densitometry tracings (– – –) after the ampholyte gel had been incubated with 70 μM RNase before [^{125}I]TyrLysDnp labeling are shown. The capacity of RNase to inhibit TyrLysDnp binding indicates the presence of immunoglobulin species that bind RNase + Dnp. Before boosting (41-day serum), most TyrLysDnp-reactive immunoglobulins in the anti-RNase population are "double-binding." After boosting with Dnp-BGG (days 52 and 77) such bands are still present. In addition, new bands now appear (*arrow*), which are not inhibited by 70 μM ribonuclease.

groups of bands appear, centered around pI 6.5 and 7.4, which bind TyrLysDnp, but are not inhibited by RNase. These bands are first seen in the animals that are bled on day 52 (10 days after boosting with Dnp-BGG) and become very strong at day 77 (25 days after boosting with Dnp-BGG). This result indicates that on boosting with Dnp-BGG, the first response is of those immunoglobulins in the anti-RNase population that happen to have anti-Dnp activity. Later on, the "primary-type" anti-Dnp response appears, consisting of bands binding TyrLysDnp which are not inhibited by RNase (see Fig. 3).

Reverse Cross-Stimulation; Immunization with Hapten B Carrier Complex and Boosting with Hapten A Carrier Complex (A = Inosine, RNase; B = Dnp). In these experiments, the primary immunization was with Dnp-BGG, and either inosine-BGG or RNase was used for the boost. The isoelectric focusing plates were developed with inosine[^{125}I]tyrosine and [^{125}I]-RNase, respectively. Inosine[^{125}I]tyrosine and [^{125}I]RNase bound strongly to their respective antibodies. These results indicate that regardless of which hapten is used for priming and which for boosting, the pattern of "double-binding" immunoglobulin bands remains the same.

Noncrossreactive Hapten Pairs. One such hapten pair, folic acid-Dnp, was studied in detail (Fig. 1*d*). Folic acid was chosen because folic acid–BGG conjugate produces a good immune response in rabbits and because the average interaction energy of folic acid with the antiserum is relatively high (15). Our results show that there are no bands in antisera against folic acid prepared in rabbits that crossreact with the Dnp haptens (Fig. 1*d*). Moreover, boosting with Dnp-BGG does not give a restricted anti-Dnp response such as is found on boosting with Dnp animals primed with uridine-BGG or inosine-BGG (Fig. 2*D*). Instead, the response closely resembles the normal primary anti-Dnp response in complexity (Fig. 2*C*).

Hapten-Carrier Control Experiments. Since the haptens used in these experiments are attached to carrier BGG, which has multiple antigenic determinants, it is possible that the carrier, rather than the hapten, is responsible both for the induction of the immunoglobulin bands that react with TyrLysDnp after the primary immunization and for the increase in these bands after boosting. When the initial immunization is done with U-BGG and the animal is boosted with Dnp-KLH (Fig. 1*d*), the isoelectric focusing spectra

closely resemble those in which the BGG was used as carrier both for the immunization and boosting step. These experiments indicate strongly that the hapten, rather than the carrier, is responsible for the cross-stimulation effects observed here.

DISCUSSION

When rabbit antihapten antibodies elicited against a hapten A are tested for their capacity to bind members of a large panel of structurally diverse antigens, a small proportion of the antigens tested show a low degree of crossreactivity. When the anti-A immune serum is resolved into immunoglobulin bands by isoelectric focusing, it is found that the ability to bind a crossreacting hapten B is the property of only a few of the many anti-A immunoglobulin bands. When the animal is boosted with the crossreacting hapten B, the early response consists principally of those immunoglobulins binding both A + B. This early "restricted" response of antibodies that bind A + B is followed by the appearance of anti-B immunoglobulin species that do not bind hapten A competitively and resemble in complexity a primary response to hapten B. We conclude that rabbit antisera against hapten contain individual immunoglobulin species capable of binding at least two structurally dissimilar haptens. These results imply that when coupled to a carrier, structurally dissimilar antigens may activate the same presumptive immunoglobulin cell-surface receptor and stimulate the production of an immunoglobulin binding both antigens. We show that the cross-stimulation is not mediated by the carrier protein. The inhibition experiments demonstrate that the immunoglobulin bands that bind A + B, which increase on boosting, do, in fact, contain single species binding A + B, and do not consist of superimposed A-binding species and B-binding species. In our experiments, 5 out of 144 haptens showed crossreactivity with an antiserum against Dnp. However, our selection of crossreactive haptens was biased by published studies. In Dnp-folic acid (a randomly chosen hapten pair) no crossreacting immunoglobulin species could be detected, and early data on screening other "noncrossreactive" hapten pairs suggest that this may be the usual situation. With small crossreactive, structurally dissimilar hapten pairs such as Dnp-inosine, Dnp-uridine, or Dnp-menadione, 3–6 crossreacting bands were detected. We know from previous work (6) that isoelectric focusing patterns of structurally "homogeneous" myeloma proteins and the products of single antibody-producing cell clones give a spectrum of 2–4 lines. It seems likely that the crossreacting bands seen with menadione-Dnp, inosine-Dnp, and uridine-Dnp could represent the product of 1–2 clones. When a complex antigen, RNase, is used as antigen A, perhaps 4–6 clones crossreact with Dnp. We known that in the myeloma protein 460, two structurally diverse haptens (Dnp + Men) bind competitively to different subsites within the combining region (3) and that these subsites are separated by 17 ± 3 Å (Manjula, B. N., Richards F. F. & Rosenstein, R. W., in preparation). The variety of structures shown here to crossreact with anti-Dnp immunoglobulins suggest that these proteins may also contain spatially separated subsites. Also, the finding that protein 460 anti-idiotype serum reacts with four "double-binding" immunoglobulin bands in our Dnp-Men system, suggests the presence of F_v regions which are related to those of protein 460 (Varga *et al.*, in preparation). There are several other suggestions that antibodies might bind more than one type of antigen (16–19).

The term "structurally diverse molecules" is, of course, relative; most small molecules have shared, as well as distinct, features and it seems possible *a priori* that attachment to the immunoglobulin could be through either. We view the antibody-combining region as a large three-dimensional array of amino-acid residues in which various permutations of contact residues could serve as specific combining sites for different antigens. Such sites could either be overlapping or spatially separated. It seems likely from our results that attachment of hapten to more than one contact site of cell-surface receptor immunoglobulin can induce production of that immunoglobulin. The specificity of the immune response is probably due to the fact that antigen selects the antibody-producing cell and causes it to divide and produce antibody. An antigen A will induce several different immunoglobulins having the ability to bind A. Such immunoglobulins will also bind a series of other antigens, but these will not be the same from immunoglobulin to immunoglobulin and will therefore be diluted out in the immune serum (1, 3, 4). If this picture is true, it becomes easy to understand the need for a complex immune response in which many groups and subgroups of antibodies are directed against a single antigenic determinant. This model has recently been subjected to combinatorial analysis (20); it was found that over a wide range of hapten number, and number of hapten specificities per site, 10^4–10^6 $V_H \times V_L$ pairs are all that is required to populate highly specific immune sera directed against an almost infinite number of haptens. Williamson and his colleagues (5, 6) have measured the number of immunoglobulin clones directed against a single determinant and have suggested the existence of polyfunctional combining regions. After immunization with antigen A and subsequent boosting with crossreactive haptens B, there is presumably an increase in the number of cells in the clones producing antibodies that bind A + B. Later, antibodies appear that bind B, but not A. These phenomena may underlie "maturation" of the humoral immune response. Early in the response, there are relatively fewer antibodies of low average intrinsic binding constant (K_0). Later on, there are more antibodies having a higher average K_0. The early response could be due to stimulation of a relatively low number of crossreactive clones raised against "natural" antigens (A) to which the animals were previously exposed. These would include both common pathogens and saprophytic bacterial flora. Since such antibodies were not initially selected for their ability to bind antigen B, it might be expected that their average K_0 for B should be low. When this A + B response is succeeded by a "primary" anti-B response, the K_0 of the immunoglobulin population with respect to antigen "B" would be expected to rise and there would be more anti-B immunoglobulin species. Direct proof of this hypothesis will require K_0 measurements of immunoglobulins reactive to both A + B and B in model systems. It may be that the cause of antibody maturation is the time-dependent decrease in antigen concentration which provides, late in the immune response, a selective advantage for cells that have surface receptors of high avidity for antigen (21). The mechanism we propose could either be synergistic with such an antigen-driven maturation mechanism or it could operate independently.

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Correction. In the article "Absence of the Sequence G-T-ψ-C-G-(A)- in Several Eukaryotic Cytoplasmic Initiator Transfer RNAs," by Simsek, M., Ziegenmeyer, J., Heckman, J. & RajBhandary, U. L., which appeared in the April 1973 issue of *Proc. Nat. Acad. Sci. USA* **70**, 1041–1045, the following correction should be made. On page 1042, line 18 from the bottom of column 1 should read: "..., 0.01 unit of purified *E. coli* alkaline phosphatase was"; not "..., 5 units...."