Proc. Nat. Acad. Sci. USA
Vol. 69, No. 1, pp. 228–232, January 1972

Homogeneous Antibody Elicited with Dinitrophenyl-Gramicidin-S

(rabbit/isoelectric focusing)

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Communicated by Herman N. Eisen, November 12, 1971

ABSTRACT **Rabbit antibody to dinitrophenyl hapten has been elicited by hyperimmunization with a bifunctional cyclic decapeptide antigen, di-dinitrophenyl-gramicidin-S. Examination of the induced antibody by isoelectric focusing, and Sipsian analysis of the hapten-binding data, demonstrated that di-dinitrophenyl-gramicidin-S was capable of stimulating and maintaining the production of a biosynthetically homogeneous antibody in the adult animal.**

Antibodies prepared against a single haptenic grouping substituted on a large multistructured carrier molecule display several types of heterogeneity (1). This heterogeneity has hampered attempts to define the nature of the antibody combining site and the origin of antibody diversity. One step in overcoming the structural ambiguities resulting from antibody heterogeneity has been the study of homogeneous myeloma proteins with antibody activity (2, 3). The myeloma proteins are the products of random pathologic events; this randomness imposes limitations on their usefulness.

Structurally restricted anti-hapten antibodies have been elicited with a heterogeneous presentation of a haptenic group in a small proportion of adult rabbits (4) and in a higher proportion of neonatal rabbits (5). Restricted heterogeneity also has been observed in the early anti-Dnp antibody response to Dnp-bovine IgG-globulin (BγG) administration, but the restriction is not maintained with further immunization (6).

A number of attempts have been made to induce homogeneous antibodies by variation of the antigen. Bacterial vaccines have induced high titers of antibody specific for bacterial polysaccharides (7, 8). These antibodies are more restricted than anti-protein antibodies; occasionally, rabbits have produced large quantities of apparently homogeneous antipolysaccharide antibody (9). The stimulation of antibody of limited heterogeneity has been attributed to the simple repeating structure of the polysaccharide antigen. Exposure to a haptenic group in a less complex structural environment has induced a low level of anti-hapten antibody exhibiting functional and electrophoretic restriction (10, 11). In addition, the polypeptide hormones bradykinin (12) and vasopressin (13) have induced antibodies of restricted heterogeneity in rabbits.

In the present study, we report the induction and maintenance of homogeneous anti-Dnp antibody in the adult rabbit by immunization with $(Dnp)_2$-gramicidin-S, a cyclic decapeptide antigen, complementary in size to the antibody combining site, carrying two identical Dnp determinants.

Abbreviations: Dnp, 2,4-dinitrophenyl hapten; BγG, Bovine IgG-globulin; [I]DDL, α-*N*-(3,5-diiodo-4-hydroxyphenacetyl)-ϵ-*N*-(2,4-dinitrophenyl)-L-lysine.

MATERIALS AND METHODS

Preparation of $(Dnp)_2$-gramicidin-S. Gramicidin-S from *Bacillus brevis* (Mann Research Laboratories) was dissolved in 10% acetic acid and filtered through a 0.9 × 150 cm column of Sephadex G-25 (Pharmacia) in the same solvent. The effluent was monitored at 258 nm, and gramicidin-S was recovered as a single component, lyophilized, and dried over P_2O_5 under reduced pressure. Amino acid analysis of the chromatographically purified gramicidin-S gave equal molar ratios of proline, valine, ornithine, phenylalanine, and leucine. No other component was detected.

Gramicidin-S was dinitrophenylated by two methods. In the first, 500 mg of gramicidin-S was dissolved in 100 ml of 95% ethanol and filtered through Whatman #50 paper. 10 g of K_2CO_3, 25 ml of redistilled water, and 5 ml of 2,4-dinitrofluorobenzene (Eastman Organic Chemicals) were added to the gramicidin-S ethanol solution in a light-tight container, and the mixture was stirred at room temperature for 24 hr. An additional 5 ml of 2,4-dinitrofluorobenzene was added and the reaction continued for another 24 hr. Dinitrophenylated gramicidin-S precipitated from the reaction mixture because of its decreased solubility in ethanol (14); the precipitate was washed repeatedly with redistilled water and cold 95% ethanol. The $(Dnp)_2$-gramicidin-S was recrystallized 3 times from boiling ethanol. In the second method, 100 mg of gramicidin-S was suspended in 10 ml of 2% (w/v) K_2CO_3 in water. 100 mg of 2,4-dinitrofluorobenzene was added to the suspension and the reaction was allowed to proceed overnight at room temperature with constant stirring in a light-tight container. The insoluble material was removed by centrifugation and sequentially washed with twice-distilled water, 50% ethanol, 100% ethanol, diethyl ether, and water. Dinitrophenylated gramicidin-S was dried and stored at 4°C under reduced pressure over P_2O_5. The extent of dinitrophenylation was determined spectrophotometrically (J. H. Rockey, P. C. Montgomery, and A. C. Bello, manuscript in preparation) and by amino acid analysis, assaying for residual ornithine. A detailed nuclear magnetic resonance (NMR) and circular dichroism study of the $(Dnp)_2$-gramicidin-S antigen has been completed in collaboration with Dr. Jim Feeney and will be published elsewhere. The NMR data furnished an added parameter for assessment of the extent of dinitrophenylation. The preparations of Dnp-gramicidin-S used for immunization in the present studies contained 1.8–2.0 Dnp groups per gramicidin-S molecule.

Preparation of anti-Dnp Antibody. Five rabbits (New Zealand reds and whites) were hyperimmunized by multiple

subcutaneous injections of 2-6 mg of $(Dnp)_2$-gramicidin-S emulsified in complete Freund's adjuvant, administered at biweekly intervals. The rabbits were bled 3–10 days after the administration of each antigen. The skin of a second group of 6 rabbits was painted with a 0.5% solution of 2,4-dinitrofluorobenzene in 50% olive oil–50% acetone (15). 20 days after skin painting, the rabbits were hyperimmunized with $(Dnp)_2$-gramicidin-S; the same schedule as that of the first group was followed. Polydispersed rabbit anti-Dnp antibody was obtained by hyperimmunization of New Zealand white rabbits with Dnp-BγG in complete Freund's adjuvant. The selection of a single anti-Dnp antibody-forming cell clone and its propagation in syngeneic mice has been described (16).

Hapten Binding Curves. α-*N*-(4-hydroxyphenacetyl)-ϵ-*N*-(2,4-dinitrophenyl)-L-lysine (DDL) (17, 18) was obtained from Doctors N. A. Mitchison and G. M. Iverson and radiolabeled with carrier-free ^{131}I or ^{125}I by the method of Hunter and Greenwood (19) to give [^{131}I]DDL or [^{125}I]DDL (5, 16-18). The labeled products were separated from excess reactants by Sephadex G-25 gel filtration in 0.15 M NaCl–50 mM borate buffer (pH 8.4) (18). Hapten binding curves obtained by radioimmunoprecipitation (13, 20) were constructed by addition of 100 μl of 10^{-5}-10^{-9}M [^{131}I]DDL (10 Ci/mmol) or [^{125}I] DDL (3 Ci/mmol) to 50 μl of anti-Dnp serum, normal rabbit serum, or an appropriate dilution of antiserum or serum. The mixtures were incubated at 4°C for 30 min, 300 μl of 75% saturated $(NH_4)_2SO_4$ was added to each mixture, the incubation at 4°C was continued for 1 hr, and the reaction tubes were centrifuged for 2 hr at 750 $\times$ *g* at 4°C. The supernatants were removed, and the precipitates, containing radio-iodinated ligand bound to anti-Dnp antibody, were carefully drained. The radioactivity of the precipitates and of the supernatants was determined in a Packard model 5219 automatic gamma counter at an efficiency of 55.2 and 30.8%, respectively, for ^{125}I and ^{131}I. The anti-Dnp serum ligand-binding data were corrected for radioactivity included nonspecifically in the precipitates, by subtraction of the radioactivity of precipitates obtained with diluted normal rabbit serum in the presence of the same ligand concentrations. The binding data were analyzed in terms of r and c, where r is the moles of ligand bound per mole of bivalent antibody ($n = 2$), and c is the free ligand concentration (here taken as the total ligand concentration minus the ligand bound specifically to the antibody). The molar concentration of antibody combining sites was obtained by plotting the ratio [bound hapten concentration]/[free hapten concentration] versus [bound hapten concentration], and extrapolation of the data to infinite free hapten concentration (13). The data were analyzed by the method of least squares in terms of the Sips distribution function, $\log [r/(n - r)] = a \log c + a \log K_0$, where a is the index of heterogeneity and K_0 is the average intrinsic association constant (13).

Anti-Dnp antibody was purified from rabbits hyperimmunized with Dnp-BγG, by affinity chromatography on a column of ϵ-N-Dnp-lysine-Sepharose 2B; the antibody was used for both radioimmunoprecipitation and equilibrium dialysis ligand-binding studies. A 20-fold range of purified anti-Dnp antibody was added to normal rabbit serum and these mixtures were used to construct hapten-binding curves obtained by radioimmunoprecipitation (see above).

Isoelectric (pI) Spectra of Anti-Dnp Antibodies. Aliquots (40-300 μl) of each serum sample were analyzed by isoelectric focusing with Ampholine carrier ampholytes (pH range 5–8, LKB) in thin layers of 5% polyacrylamide gels (5, 18, 21). The focusing (final potential 500 V) was continued for 24 hr, the gel was overlayed with a 1 μM solution of [^{131}I] DDL or [^{125}I] DDL, developed at 37°C for 15 min, and fixed in 18% (w/v) Na_2SO_4 in water. The gels were washed, dried, and a contact autoradiograph was made on an x-ray film (Ilfex, Ilford Limited).

RESULTS

Three of the 6 rabbits immunized with $(Dnp)_2$-gramicidin-S after skin painting with 2,4-dinitrofluorobenzene, and 2 of 5 rabbits immunized without prior skin painting, developed anti-Dnp antibodies. The antibodies, demonstrated by radioautography after isoelectric focusing, showed a markedly restricted number of isoelectric bands. The patterns were strikingly similar to the isoelectric (pI) spectra of purified mouse IgG myeloma proteins from serum (22, 23), and of a biosynthetically homogeneous murine IgG anti-Dnp antibody produced by a single clone of antibody-forming cells selected in irradiated syngeneic CBA mice (Fig. 1) (ref. 16). One rabbit (R13) was selected for more detailed study. An autoradiograph of the R13 anti-Dnp antibody, elicited by immunization of an adult rabbit with $(Dnp)_2$-gramicidin-S after skin painting, is presented in Fig. 1. The isoelectric spectra of the biosynthetically homogeneous murine anti-Dnp antibody and of polydispersed (polyclonal) rabbit antibody induced with Dnp-BγG also are illustrated in Fig. 1.

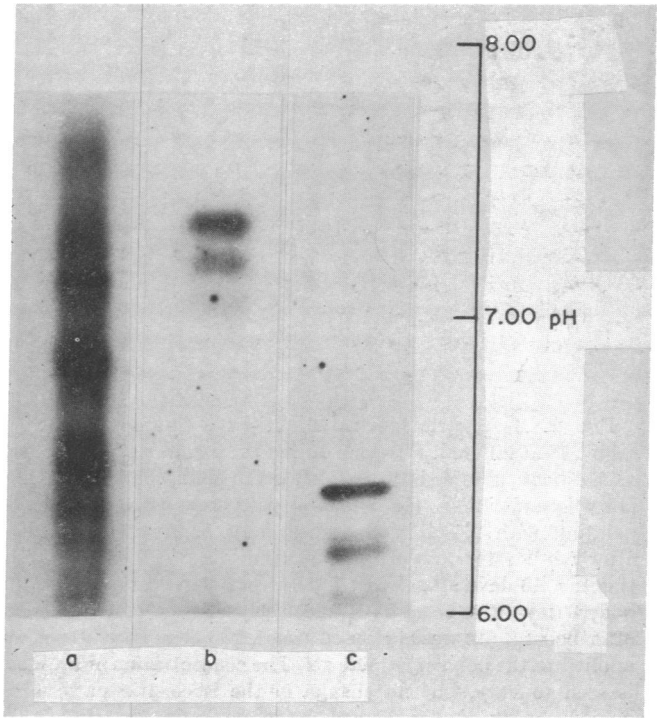

FIG. 1. Isoelectric spectra of anti-Dnp antibodies. *a*, Polydispersed (polyclonal) rabbit antibody induced with Dnp-BγG. *b*, Rabbit antibody induced with $(Dnp)_2$-gramicidin-S. *c*, Monoclonal murine antibody induced in CBA mice with Dnp-BγG and selected by cloning antibody-forming cells in irradiated syngeneic mice (15). Anti-Dnp antibodies have been specifically labeled, after isoelectric focusing, by binding [^{131}I]DDL; the distribution of the radioactive label has been demonstrated by contact autoradiography.

R13 was maintained on the boosting schedule with $(Dnp)_2$-gramicidin-S in complete Freund's adjuvant. Sera from successive bleedings showed increased amounts of anti-Dnp molecules with the same isoelectric spectrum (Fig. 2). Since identical volumes of serum were focused on each track in the gel of Fig. 2, the relative intensities of the bands on the autoradiograph reflect the concentration of antibody in serum at each bleeding. The anti-Dnp antibody concentrations of the serum samples examined in Fig. 2*a,b,c,d,* and *e,* determined from hapten-binding data obtained by radioimmunoprecipitation, were 22, 75, 12, 45, and 40 μg/ml, respectively. The low amount of anti-Dnp antibody in the third serum sample (Fig. 2*c*) reflects the decrease in production that occurred during a rest period in the immunization schedule. The subsequent injections of antigen increased the level of anti-Dnp antibody without increasing the heterogeneity of the isoelectric spectrum (Fig. 2*d* and *e*). The apparent variation in the number of bands (3-4) seen with different serum samples in Fig. 2 was due to variations in antibody concentration. This was established by subjecting a range of volumes of each serum sample to isoelectric focusing, or by developing the radioautographs for prolonged periods, and observing that a maximum of 4 bands was obtained with each serum sample. Higher concentrations (100-340 μg/ml) of anti-Dnp antibody of severely restricted isoelectric heterogeneity have been demonstrated more recently in other rabbits immunized with $(Dnp)_2$-gramicidin-S.

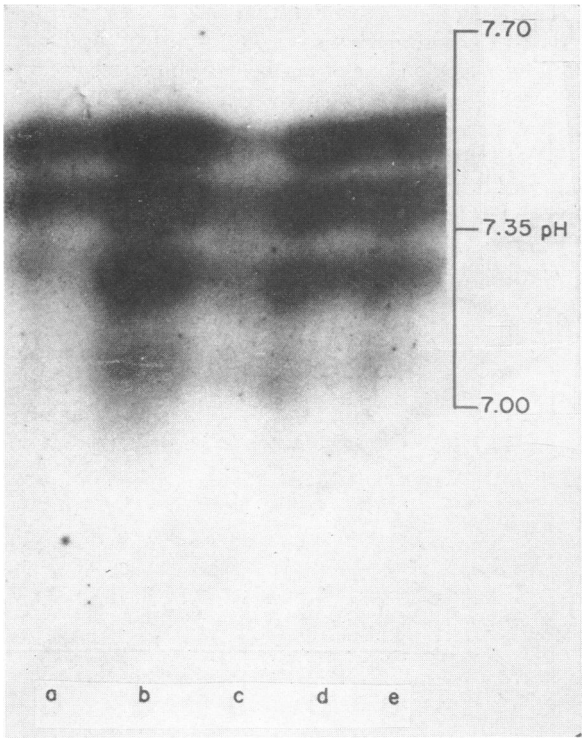

FIG. 2. Isoelectric spectra of anti-Dnp antibody elicited in rabbit 13 at different intervals during the immunization schedule. *a,* Antibody obtained 10 days after the sixth administration of $(Dnp)_2$-gramicidin-S (14 weeks elapsed time after initial skin painting with 2,4-dinitrofluorobenzene); *b,* Antibody 7 days after 7th booster dose of $(Dnp)_2$-gramicidin-S (23 weeks elapsed time); *c,* 46 days after boost 7 (30 weeks elapsed time); *d,* Antibody 3 days after boost 8 (31 weeks elapsed time); and *e,* 10 days after boost 8 (32 weeks elapsed time). 100 μl of each serum was applied to the polyacrylamide gel. The concentration of antibody is seen to vary, but no change in the isoelectric pattern occurred during the prolonged immunimization schedule. The apparent loss of the weakest band at pH 7.1 in *a* and *c* was a reflection of low antibody concentrations in these sera. The anti-Dnp antibody concentrations in these sera were: *a,* 22 μg/ml; *b,* 75 μg/ml; *c,* 12 μg/ml; *d,* 45 μg/ml; and *e,* 40 μg/ml. Additional rabbits have displayed similar isoelectric focusing spectra. Mixing experiments have shown slight differences in the isoelectric points of individual bands, while the total number of spectral components and spread of isoelectric points remained analogous to those in the serum from rabbit 13.

The isoelectric focusing radioautography is capable of detecting 0.1 pg or more of anti-Dnp antibody per isoelectric band (18).

The Sipsian plots of the hapten-binding data obtained for the three antibodies of Fig. 1 by radioimmunoprecipitation are shown in Fig. 3. The heterogeneity of the antibody directed against Dnp-BγG is reflected in a heterogeneity index value of 0.63. The heterogeneity index values obtained for the murine monoclonal Dnp-BγG antibody and the $(Dnp)_2$-gramicidin-S antibody both were unity, reflecting their lack of association constant heterogeneity.

The association constant (K_0) and index of heterogeneity (a) obtained for a single purified anti-Dnp antibody preparation when added to normal serum and determined by radioimmunoprecipitation were, within the limits of experimental error, identical to those values obtained by equilibrium dialysis. These results will be presented in more detail elsewhere (P. C. Montgomery and A. R. Williamson, manuscript in preparation). A 20-fold variation in the concentration of

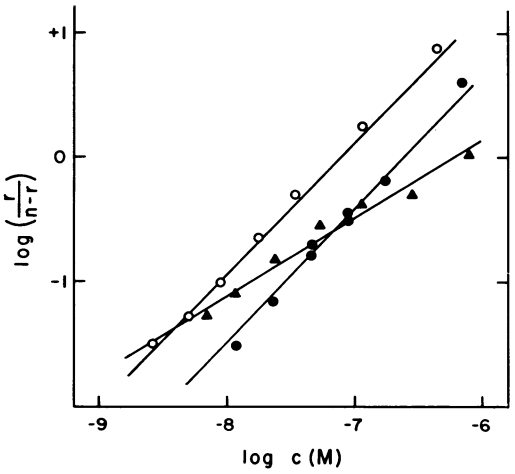

FIG. 3. Hapten binding curves constructed with [^{125}I]DDL by the modified Farr technique. Binding data are expressed in terms of the Sips distribution function, $\log [r/(n - r)] = a \log c + a \log K_0$, where r is the moles of hapten bound per mole of antibody, c is the free hapten concentration, K_0 is the average intrinsic association constant, and a is the index of heterogeneity. The valence of the antibody, n, was taken as 2. The molar concentration of antibody combining sites was determined by plotting the data in the form [bound hapten]/[free hapten] versus [bound hapten] and extrapolating the data to infinite concentration of free hapten. The association constants and the indexes of heterogeneity obtained from these data are presented in Table 1. (○—○) monoclonal murine anti-Dnp-BγG, (●—●) monoclonal rabbit anti-$(Dnp)_2$-gramicidin-S, (▲—▲) polyclonal rabbit anti-Dnp-BγG.

added purified anti-Dnp antibody did not alter the association constant and heterogeneity index values, obtained by radioimmunoprecipitation in the presence of normal serum proteins.

Table 1 summarizes the average intrinsic association constants and the heterogeneity indexes obtained for the three antibodies. These data were obtained directly from the binding data presented in Fig. 3. The association constant and the index of heterogeneity for R13 anti-$(Dnp)_2$-gramicidin-S antibody remained the same throughout the immunization schedule.

TABLE 1. *Association constants (K_0) and heterogeneity indices (a) of anti-Dnp antibodies*

Anti-Dnp antibody	K_0* ($M^{-1} \times 10^{-6}$)	a†
Polyclonal rabbit anti-Dnp-BγG	1.6	0.63
Monoclonal murine anti-Dnp-BγG‡	12.6	1.05
Monoclonal rabbit anti-$(Dnp)_2$-gramicidin-S	3.8	1.05

* Average intrinsic association constant determined by radioimmunoassay with [^{125}I]DDL (Fig. 3).

† Heterogeneity index obtained from the Sips plot of Fig. 3.

‡ Selected by cloning in irradiated syngeneic mice (16).

DISCUSSION

The heterogeneous substitution of the Dnp determinant on a complex protein carrier such as bovine IgG-globulin and immunization with this antigen, in a majority of instances, results in the production of heterogeneous antibody (5, 6, 10). The heterogeneity of one such antibody preparation is illustrated by the isoelectric spectra in Fig. 1. In marked contrast, the antibody directed against $(Dnp)_2$-gramicidin-S displayed a highly restricted isoelectric spectrum (Fig. 1). This restricted isoelectric spectrum must be interpreted in terms of the multiple band patterns characteristic of monoclonal immunoglobulins resulting from multiple myeloma (22, 23) and the band patterns produced by the anti-Dnp antibody of a single antibody-forming cell clone selected in CBA mice (Fig. 1, ref. 16). The simplicity of the antibody spectrum elicited by $(Dnp)_2$-gramicidin-S may in fact represent the phenotype of a single clone.

Anti-Dnp antibody with a similarly restricted isoelectric spectrum was elicited in adult rabbits by hyperimmunization with $(Dnp)_2$-gramicidin-S either with or without prior skin painting with 2,4-dinitrofluorobenzene, indicating that prior Dnp sensitization was not a necessary condition for the response.

It is important to note that the initial homogeneity reflected in the isoelectric focusing pattern has been maintained for at least ten months. It is unlikely that this continued production of a single anti-Dnp antibody is due to the appearance of a neoplastic cell clone, since the response is antigen dependent (Fig. 2).

The heterogeneity index of unity calculated from the binding data also reflects the homogeneity of the anti-$(Dnp)_2$-gramicidin-S antibody. The occurrence of a single association constant and a unitary index of heterogeneity throughout the immunization schedule for R13 supports the contention that the product of but a single cell clone is being examined.

The isoelectric spectrum yields information on the number of different antibody molecules and their proportional representation in serum. The multicomponent spectrum of the rabbit anti-Dnp-BγG antibody (Fig. 1) results from the fact that these antibodies have structural differences that are reflected in distinct isoelectric points. Therefore, this technique directly assesses structural heterogeneity. The possible but unlikely occurrence of two or more distinct antibody populations possessing the same isoelectric point and therefore appearing structurally homogeneous is not fully ruled out. It is, however, likely that such a set of antibodies would possess different affinities that would be reflected in the heterogeneity index measurement. An exact correlation between the complexity of the isoelectric spectrum and the heterogeneity index is not expected since the two methods measure different parameters (5). However, when an antibody displays a monoclonal isoelectric spectrum and also has a heterogeneity index of one, it may be considered biosynthetically homogeneous. The anti-$(Dnp)_2$-gramicidin-S antibody studied here satisfies these criteria. Amino-acid-sequence studies, as well as chain recombination experiments, should provide final confirmation of homogeneity.

The antigen, $(Dnp)_2$-gramicidin-S, possesses a rigid, stable, cyclic structure with a 2-fold axis of symmetry, carrying two Dnp groups in an identical conformational environment (J. H. Rockey and J. Feeney, manuscript in preparation). These characteristics appear to contribute to its usefulness in eliciting homogeneous anti-Dnp antibody. The bifunctional nature of the antigen is of interest in considering the possibility that two cooperating cell populations, a thymus-derived Dnp-specific lymphocytic cell line and a bone marrow-derived Dnp-specific cell line, are involved in the production of antibody. We are now examining monosubstituted Dnp-gramicidin-S as an antigenic determinant. In addition, gramicidin-S is being used as a carrier for other haptenic groups.

The authors gratefully acknowledge the excellent technical assistance of Miss A. Bello, Mrs. C. Garnett, and Miss C. Steenstra. This work was supported in part by the National Institute of Dental Research USPHS DE-02623 and Research Grant AI-05305 from the National Institute of Allergy and Infectious Diseases, USPHS.

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