

SPATIAL REQUIREMENTS BETWEEN HAPTENIC  
AND CARRIER DETERMINANTS FOR  
T-DEPENDENT ANTIBODY RESPONSES\*

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The immunogenicity in guinea pigs and mice of the small molecule L-tyrosine-*p*-azobenzene-*o*-arsenate (RAT) and several of its analogues have proven extremely useful for investigating a number of parameters of the immune response, particularly the antigen structural requirements for lymphocyte activation and for cell cooperation (1, 2). The unideterminant antigen induces cellular immunity without discernible antibody formation (3-5), even by the extremely sensitive passive neutralization of bacteriophage assay (6). This accords with expectations in that unideterminant antigens, though capable of interacting with specific T and B lymphocytes, should be unable to mediate cooperation between them.

On the other hand, it would be anticipated that bifunctional antigens composed of one RAT moiety serving as a carrier and a second group serving as a hapten should behave as complete antigens and induce antibody responses as well as cellular immunity. This proved to be the case whether the haptenic component was multivalent-poly-D-glutamic acid with an average mol wt of 35,000 (3), or a single dinitrophenyl group (7). In either case, antibody specific for the hapten was produced, whereas T-cell specificity, assessed by delayed hypersensitivity, lymphocyte transformation, and carrier-induced helper activity, was directed against the RAT moiety. The findings have been in general accord with simple antigen-bridging models of cell cooperation (Fig. 1).

Separating the RAT carrier determinant and 2,4-dinitrophenyl (DNP) haptenic determinant of bifunctional molecules with spacers of varying size permitted an assessment of the spatial requirements between hapten and carrier for an anti-hapten response (7). The spacer used for this purpose was 6-aminocaproic acid (SAC), a flexible chain of six carbon atoms with an extended span of 8 Å. One or more spacers could be coupled in stepwise fashion to tyrosine, leaving the amino group of the terminal spacer available for substitution with DNP. The magnitude of the anti-DNP response was similar whether the determinants were separated by one or three SAC spacers, but was significantly weaker when the determinants were joined without a spacer. Thus, cooperation could apparently be implemented by an antigen in which the carrier and haptenic moieties were separated by <8 Å.

The abundant evidence that helper T cells modulate the antibody response by

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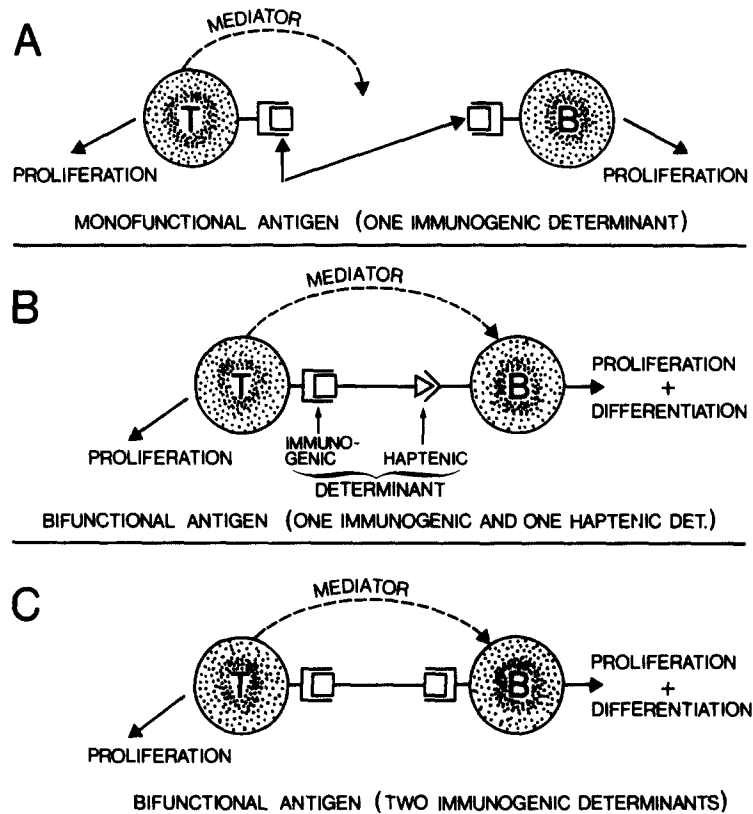


FIG. 1. Models of cooperation between T and B cells in response to: (A) a monofunctional immunogen such as RAT; (B) an asymmetric bifunctional antigen such as DNP-SAC-RAT; (C) a symmetrical bifunctional antigen such as RAT-(PRO)<sub>10</sub>-RAT (1). The role of macrophages has been omitted.

elaborating soluble mediators which serve as activating signals for B cells (8), together with the firmly established requirement for linkage between hapten and carrier to obtain secondary anti-hapten responses, argues that the mediator can trigger B cells only in the immediate vicinity of activated T cells, which probably reflects the need for a threshold concentration of the factor. This proximity requirement is even more striking when it is considered that lymphocyte activation probably takes place at the surface of macrophages (9), which are not selective for antigen. Thus, the same cells would be expected to take up and express hapten and carrier even when the two were introduced as separate molecular entities, yet no anti-hapten responses ensue under those circumstances. These observations lead to the prediction that bifunctional antigens might be constructed with rigid spacers of sufficient dimension to obviate the delivery of an effective T-cell signal to the appropriate B cell. Rigid spacers would be required for this purpose to prevent folding of the spacer chain with consequent indeterminate variance of the distance between the carrier and haptenic epitopes. If such proved to be the case, estimates could be made of the maximum permissible distance between T and B cells for activation of the latter, based again on the antigen-bridging model. The present communication describes our approach to the resolution of this question.

### Materials and Methods

**Antigens.** Bifunctional antigens with rigid spacers (DNP-[proline, PRO]<sub>n</sub>-RAT) were synthesized by the solid-phase technique (10) on a chloromethylated styrene polymer (Lab Systems, San Mateo, Calif.), 1% cross-linked with divinylbenzene. The cesium salt of *N*-tert-butyloxycarbonyl(BOC)-0-2,6-dichlorobenzyl-L-tyrosine (Bachem, Torrance, Calif.) was esterified to the polymer (11) and proline chains were added by repeated attachment of the tripeptide derivative *N*-BOC-(PRO)<sub>3</sub>-OH. This tripeptide was synthesized by coupling *N*-BOC-L-proline with L-proline benzyl ester. The crystalline dipeptide was deprotected by hydrogenolysis to yield crystalline *N*-BOC-(L-PRO)<sub>2</sub>-OH. This product was reacted again with L-proline benzyl ester to yield an oily protected tripeptide ester. Hydrogenolysis gave the desired tripeptide in crystalline form.

To avoid the chain termination observed during synthesis of proline peptides (12), the chain elongation steps were carried out using a threefold excess of symmetrical anhydrides preformed by dicyclohexylcarbodiimide (13). Deprotection was carried out with trifluoroacetic acid and triethylamine was used for neutralization. Synthesis was concluded at the desired chain length by coupling DNP-PRO as the terminal residue. The peptides were cleaved from the polymer by liquid hydrogen fluoride (14).

A small amount of polymer was cleaved after each coupling step during synthesis to analyze the products by high voltage electrophoresis. An unknown byproduct was observed to accumulate during the synthesis, particularly during the (PRO)<sub>30</sub>-tyrosine(TYR) to (PRO)<sub>39</sub>-TYR steps. The byproduct was electrophoretically neutral at pH 1.85 and failed to react with isatin or ninhydrin. It was easily separated from the positively charged, amino-containing correct peptide. The product is not simply *N*-trifluoroacetyl peptides, which have been observed previously (12). Its identity is being investigated.

The cleaved peptides were purified on Sephadex G-15 and LH-20 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) columns in water. Each was then reacted with diazotized arsanilic acid and the DNP-(PRO)<sub>n</sub>-RAT peptides were finally purified by chromatography on Sephadex G-15, LH-20, and LH-60 columns in water. A more detailed description of the synthesis will be published elsewhere.

**Animals and Immunization.** Outbred guinea pigs were injected in the footpads with 0.24, 1.0, or 4.0  $\mu$ mol of DNP-(PRO)<sub>n</sub>-RAT conjugates emulsified in complete Freund's adjuvant. Sera from immunized animals were obtained by cardiac puncture.

**Quantitative Precipitin Determinations.** Bovine serum albumin (BSA) conjugates of DNP and azobenzenearsonate (ABA), prepared as described before (7), served as antigens for the quantitation of anti-DNP and anti-ABA antibody, respectively, by a micromodification of the Folin-Ciocalteau method (15). Anti-proline antibody was assayed with synthetic poly-L-proline (Miles Laboratories, Kankakee, Ill.).

**Delayed Hypersensitivity.** Skin tests of immunized animals were performed on a shaved area of the flank by intradermal injection of 0.05 mg of ABA-BSA in 0.1 ml of saline, pH 7.2. Skin sites were examined at 2-4 h for signs of Arthus reactions and at 24 and 48 h for delayed reactions. Reactions were considered positive if they consisted of an area of induration and erythema 5 mm in diameter or larger.

### Results

The anti-DNP antibody responses of guinea pigs immunized with DNP-(PRO)<sub>n</sub>-RAT bifunctional antigens with spacers of varying dimension are summarized in Table I. The PRO<sub>10</sub> and PRO<sub>22</sub> compounds evoked responses that peaked  $\approx$  3 wk after immunization at levels of 150-200  $\mu$ g anti-DNP antibody per milliliter of serum. These responses were quantitatively and kinetically very similar to those obtained using DNP-(SAC)<sub>1-3</sub>-RAT bifunctional antigens (7). The optimal antigen dose of the three tested (0.24, 1.0, and 4.0  $\mu$ mol) was 1.0  $\mu$ mol in each instance. This dose of DNP-PRO-RAT did not raise significant anti-DNP responses and, of particular interest, DNP-(PRO)<sub>31</sub>-RAT was negative at all doses and times assayed. Extending the spacer chain to 40 proline residues also failed to provide effective bifunctionality

TABLE I  
*Antibody Responses to Bifunctional Antigens with Polyprolyl Spacers*

Antigen	Spacer* length	Dose	Anti-DNP Antibody, $\mu\text{g/ml}$ weeks after immunization				
			1	2	3	4	5
	$\text{\AA}$	$\mu\text{M}$					
DNP-PRO-RAT	3	1.0	NT $\S$	0	0	0	NT
DNP-(PRO) <sub>10</sub> -RAT	31	0.24	0	$52 \pm 25\ddagger$	$70 \pm 26$	$61 \pm 21$	NT
		1.0	NT	$142 \pm 26$	$203 \pm 50$	$64 \pm 16$	$5 \pm 5$
		4.0	0	$7 \pm 7$	$161 \pm 40$	$72 \pm 29$	NT
DNP-(PRO) <sub>22</sub> -RAT	69	0.24	0	$85 \pm 6$	$123 \pm 8$	NT	NT
		1.0	NT	NT	$143 \pm 14$	$17 \pm 12$	$2 \pm 2$
		4.0	0	$98 \pm 9$	$117 \pm 11$	NT	NT
DNP-(PRO) <sub>31</sub> -RAT	97	0.24	0	0	0	0	NT
		1.0	0	0	0	0	NT
		4.0	0	0	0	0	NT
DNP-(PRO) <sub>69</sub> -RAT	125	1.0	0	0	0	0	NT

\* Based on axial translation of 3.12  $\text{\AA}$  per proline residue in poly-L-proline (16, 17).

$\ddagger$  Arithmetic mean  $\pm$  SE, three to six guinea pigs per group.

$\S$  Not tested.

at a dose of 1.0  $\mu\text{mol}$ , indicating that the failure of DNP-(PRO)<sub>31</sub>-RAT was not idiosyncratic.

None of the animals developed detectable anti-ABA antibody, although they regularly developed delayed skin reactivity when tested with ABA-BSA, in accord with findings reported earlier for bifunctional antigens with flexible spacers (7). Some animals developed small quantities of anti-polyprolyl antibody, but there were no significant differences between the groups. Thus, animals immunized with 1.0  $\mu\text{mol}$  of the (PRO)<sub>10</sub>, (PRO)<sub>22</sub>, and (PRO)<sub>31</sub> compounds made  $23 \pm 23$ ,  $31 \pm 23$ , and  $16 \pm 14$   $\mu\text{g}$  of anti-polyproline antibody per milliliter of serum, respectively. These responses are generally similar to those made to a RAT-(PRO)<sub>10</sub>-RAT symmetrical bifunctional antigen (12).

### Discussion

The present findings, interpreted in the context of the antigen-bridging model of cell cooperation (Fig. 1), indicate that the cut-off distance between the interacting cells for delivery of an effective T-cell signal to the B cell lies between 69 and 97  $\text{\AA}$ . Aside from the validity of the model, which rests on its consistency with a large body of experimental data (Introduction), this conclusion depends on several assumptions concerning the rigidity and dimension of polyproline chains.

Polyproline exists in two alternative forms (16, 17). Form I is a *cis* right-handed helix with an axial translation of 1.9  $\text{\AA}$  per residue. This conformation is assumed in nonpolar solvents such as pyridine or aliphatic alcohols. Form II is a *trans* left handed helix with three residues per helical turn and an axial translation of 3.12  $\text{\AA}$  per residue. The *trans* conformation is assumed in aqueous (polar) solvents. We used the form II coordinates in our calculations of the distances between the haptenic and carrier determinants of the bifunctional molecules. The reliability of the figures is supported by several studies. Schimmel and Flory (18) have shown that the mean square end-to-end distance of a hexadecamer of L-proline agrees very closely with the value obtained from the Cowan and McGavin (17) helix coordinates for form II (18). For longer chain lengths (circa 50 residues), intrinsic viscosities show excellent agreement with predicted values (19). Moreover, in studies more closely related to the present one, in

which oligomers of poly-L-proline were used as spacers to separate donor and acceptor chromophores in energy transfer measurements, the observed efficiency of energy transfer as a function of distance was also in excellent agreement with predictions (20). These observations, in aggregate, provide solid support for the assumption that chains of polyproline with 10–50 residues behave as rigidly extended rods in solution.

An abrupt abrogation of the anti-DNP response resulted from increasing the spacer size from 22 to 31 proline residues, corresponding to a dimensional increment of 28 Å U (69 as compared to 97 Å). The maximum permissible separation could, of course, be delineated more precisely by fabricating antigens with intermediate spacer chains. Moreover, the model could be tested further by constructing antigens with nonpermissive polyproline spacers (e.g., 31 residues) in which another amino acid was substituted for proline at one position in the chain, permitting a bend or kink in the spacer. Such an antigen might be expected to induce anti-DNP antibody responses. Experiments along these lines are in progress.

### Summary

To gauge the proximity between cooperating T and B cells required for effective triggering of antibody production, guinea pigs were immunized with bifunctional antigens in which the haptenic and carrier determinants were separated by rigid spacers of varied dimension. These took the form 2,4-dinitrophenol-(proline)<sub>n</sub>-L-tyrosine-*p*-azobenzenearsonate, where *n* varied from 1 to 40 proline residues. Animals immunized with *n* = 10 and *n* = 22 compounds made strong anti-DNP antibody responses, whereas animals immunized with bifunctional compounds containing longer spacers did not make antibody detectable by precipitation. It can be calculated on the basis of very strong physicochemical evidence for the rigidity and axial translation of poly-L-proline chains in solution that the cut-off point for effective interaction between T and B cells lies between 69 and 97 Å U.

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