ANTIGEN RECOGNITION AND THE IMMUNE RESPONSE

"Self-Help" with Symmetrical Bifunctional Antigen Molecules*

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The immunogenicity of the small molecule L-tyrosine-*p*-azobenzenearsonate (RAT)[1] (1, 2) provides an ideal tool for investigating a number of parameters of the immune response. Included among these are the minimum separation between haptenic and carrier determinants required for an anti-hapten antibody response and whether "self-help," the capacity of an immunogenic determinant to cooperate in the humoral response to a second identical determinant, is possible (3). Asymmetric bifunctional molecules consisting of one RAT determinant and one dinitrophenyl (DNP) determinant separated by spacers of various size consistently induced anti-DNP antibody and cellular immunity specific for the RAT group (3). On the other hand, symmetrical bifunctional molecules composed of two RAT determinants separated by the same spacers were unable to provoke either primary or secondary anti-RAT humoral responses (3), although it is well known that the arsonate group is a potent hapten.

The spacers used in these studies were flexible chains comprised of one or more units of 6-aminocaproic acid (SAC). Since RAT contains both electropositive (azo) and electronegative (arsonate) centers, the failure of bifunctional RAT compounds with flexible spacers to induce humoral immunity could be ascribed to intramolecular association of the two determinants, in which the basic centers align with the acidic centers in a "deck of cards" geometry (4). Such intramolecular stacking would compromise the bifunctional characters of these molecules. On the other hand, it is also conceivable that both determinants might interact with receptors on the surface of a single immunocyte, which would fail to satisfy the requirement for intercellular cooperation.

In order to distinguish between these alternative possibilities, bifunctional RAT compounds containing rigid spacers and bifunctional antigens composed of

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[1] *Abbreviations used in this paper*: BSA bovine serum albumin; CFA, complete Freund's adjuvant; DNP, dinitrophenyl; RAT, L-tyrosine-*p*-azobenzenearsonate; SAC, 6-aminocaproic acid; TAT, L-tyrosine-*p*-azophenyltrimethylammonium chloride.

two L-tyrosine-*p*-azophenyltrimethylammonium (TAT) determinants separated by flexible or rigid spacers were synthesized. TAT is immunogenic in guinea pigs, does not cross-react with RAT, and contains only electropositive centers, which precludes intramolecular association (3). The findings obtained from immunization of guinea pigs with these compounds comprise the present communication.

Materials and Methods

Antigens.—Bifunctional antigens with rigid spacers were synthesized by the solid phase technique (3, 5) using polyproline as the spacer. A chain of 10 proline residues was decided upon because it has a span of about 22 A, comparable to that of the largest flexible spacer used previously (3). *N-tert*-Butyloxycarbonyl-L-tyrosine-*O*-benzyl ether, *N-tert*-butyloxycarbonyl-L-proline, and *N*-acetyl-L-tyrosine-*O*-benzyl ether were purchased from Bachem Co., Marina Del Ray, Calif. The chloromethylated styrene polymer, Bio-Beads S-X-1, 1.25 meq/g capacity, was purchased from Bio-Rad Laboratories, Richmond, Calif. Starting with the C-terminal attachment of L-tyrosine, acetyl-L-tyrosine-(L-proline)$_{10}$-L-tyrosine (Ac-L-Tyr-[L-Pro]$_{10}$-L-Tyr) was synthesized in stepwise fashion, using trifluoroacetic acid in methylene chloride for the deprotection steps. A small amount of product was removed from the polymer after each coupling step for analysis by cleavage with hydrogen bromide in trifluoroacetic acid. High voltage electrophoresis on paper at pH 1.85 indicated complete coupling at each step. However, an amino-protected acidic by-product was observed to be accumulating during the synthesis. After completion of the entire sequence, the crude product was treated with an aqueous solution of 1 N piperidine, which removes the trifluoroacetyl groups, and the by-product resolved into a series of (Pro)$_n$-Tyr peptides on electrophoresis. Thus, it consisted of trifluoroacetyl-(Pro)$_n$-Tyr peptides which formed during synthesis to an extent of 11% of acetyl-Tyr-(Pro)$_{10}$-Tyr by weight.

The desired acylated dodecapeptide was easily purified on a carboxymethylcellulose column in water and coupled with the appropriate diazonium reagent to yield either RAT or TAT derivatives (3). Since the two tyrosines in this peptide are not equivalent and each tyrosine may either not react or give mono- or bis-substituted products during diazonium coupling, nine different compounds may result. The resulting mixture was purified by column chromatography on Sephadex G-15 in 0.1 M ammonia, followed by high voltage electrophoresis on Whatman 3 MM paper at pH 6.4, elution with water, and chromatography on a small carboxymethylcellulose column in water. The purified compound gave the expected optical density at 325 mμ in 0.1 N sodium hydroxide, indicating two chromophores per molecule. In the physiologic pH range, the extinction coefficient of the compound did not exhibit hypochromism, indicating that intramolecular interactions were not taking place.

Monofunctional antigens were produced in a similar manner without the addition of N-terminal tyrosine to the synthetic product. Bifunctional acetyl-TAT-SAC-TAT was fabricated as described previously for acetyl-RAT-SAC-RAT (3). Conjugates of diazonium compounds with bovine serum albumin (BSA) were also described earlier (3). Poly-L-proline was purchased from Miles Laboratories, Inc., Kankakee, Ill.

Animals.—Randomly bred guinea pigs weighing about 600 g were used in all experiments.

Immunization.—Antigens were dissolved in 0.15 M sodium chloride (1.22 μmoles of antigen/ml of saline), the pH was adjusted to 7.0, and the aqueous solutions were emulsified with complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich.). Animals received 0.1 ml of the emulsion in each footpad. They were bled 10 and 20 days after immunization, and skin was tested on the 21st day.

Delayed Hypersensitivity.—Skin tests of immunized animals were performed on a shaved area of the flank by intradermal injection of 50–100 μg of antigen in 0.1 ml of saline, pH 7.2.

Skin sites were examined at 2–4 hr for signs of Arthus reactions and at 24 and 48 hr for delayed reactions. Reactions were considered positive if they consisted of an area of induration and erythema 5 mm in diameter or larger.

Quantitative Precipitin Determinations.—The BSA conjugates served as test antigens for detection of specific antibody. Sera were assayed using a micromodification of the Folin-Ciocalteu method for determining quantitites of protein in the range of 1–10 μg, as previously described (6).

RESULTS

Immunization of guinea pigs with Ac-RAT-$(Pro)_{10}$-RAT led to substantial quantities of precipitable anti-RAT antibody, as well as to comparable amounts of anti-prolyl antibody (Table I). Only molecules carrying the RAT group

TABLE I

Response of Guinea Pigs to Symmetrical Bifunctional RAT Antigens with Polyprolyl Spacers

Test antigens	Immunizing agent							
	Ac-RAT-$(Pro)_{10}$- RAT		Ac-$(Pro)_{10}$-RAT		Ac-Tyr-$(Pro)_{10}$-Tyr		Ac-$(Pro)_{10}$-Tyr	
	Ppt. antibody	D.S.R.*	Ppt. antibody	D.S.R.	Ppt. antibody	D.S.R.	Ppt. antibody	D.S.R.
	$\mu g/ml$	*mm*	$\mu g/ml$	*mm*	$\mu g/ml$	*mm*	$\mu g/ml$	*mm*
$(RAT)_{11}$-BSA	135 ± 18	19 (4)‡	<2	20 (4)	<2	2 (4)	<2	2 (4)
Poly-L-proline	158 ± 34	0	<2	4	<2	3	<2	4
Ac-Tyr-$(Pro)_{10}$-Tyr		0				4		
Ac-$(Pro)_{10}$-Tyr				0				4
BSA	<2	0	<2	0	<2	0	<2	0

* Average delayed skin reaction.

‡ Figures in parentheses refer to number of animals tested.

elicited delayed hypersensitivity reactions. The monofunctional RAT compound Ac-$(Pro)_{10}$-RAT did not induce anti-RAT antibody, demonstrating that $(Pro)_{10}$ lacked carrier activity, as expected, based on its inability to elicit cellular immune reactions. This compound induced cellular immunity to RAT, but did not raise significant quantities of anti-prolyl antibody, in marked contrast to the bifunctional RAT compound. The basis for this distinction is not understood since RAT can serve as a carrier for other haptens such as DNP which are directly linked to it (3). However, anti-prolyl antibody is notoriously difficult to prepare using conventional protein carriers (D. Michaeli, personal communication), and the quantities engendered by Ac-RAT-$(Pro)_{10}$-RAT were surprising. Immunization with control compounds shown in Table I which lack the RAT moiety (Ac-Tyr-$(Pro)_{10}$-Tyr and Ac-$(Pro)_{10}$-Tyr) were nonimmunogenic, as anticipated.

The series of TAT compounds shown in Table II all engendered cellular immunity to the TAT group, but only the bifunctionals provoked humoral anti-

TABLE II

Response of Guinea Pigs to Symmetrical Bifunctional TAT Antigens

Test antigens	Immunizing agent					
	TAT		Ac-TAT-$(Pro)_{10}$-TAT		Ac-TAT-SAC-TAT	
	Ppt. antibody	D.S.R.*	Ppt. antibody	D.S.R.	Ppt. antibody	D.S.R.
	μg/ml	*mm*	*μg/ml*	*mm*	*μg/ml*	*mm*
$(TAT)_5$-BSA	<2	19 (4)‡	189 ± 32	12 (4)	74 ± 13	20 (3)
Poly-L-Proline			26 ± 10	0		
Ac-Tyr-$(Pro)_{10}$-Tyr				0		
$(RAT)_{11}$-BSA		0		0		0

* Averaged delayed skin reaction.

‡ Figures in parentheses refer to number of animals tested.

TAT responses. In contrast to Ac-RAT-SAC-RAT, which failed to stimulate either primary or secondary anti-RAT responses (3), Ac-TAT-SAC-TAT elicited significant levels of anti-TAT antibody. Like the RAT and TAT bifunctionals with prolyl spacers, this compound failed to demonstrate a hypochromic effect spectrophotometrically.

DISCUSSION

Monofunctional immunogenic molecules such as RAT (1, 2) and TAT induce cellular immunity without significant amounts of humoral antibody. Nonsymmetrical bifunctional antigens bearing one immunogenic (carrier) determinant and one nonimmunogenic (haptenic) determinant provoke cellular immunity to the carrier determinant and humoral immunity primarily directed against the haptenic component (3). The present findings demonstrate that symmetrical bifunctional molecules bearing identical immunogenic determinants also induce cellular and humoral immunity. We have designated this phenomenon self-help, although it is not intended to imply that the cellular and humoral components have identical specificity, but merely that they are specific for structural aspects of the same determinant.

A complicating factor in the interpretation of experiments using bifunctional antigens with polyprolyl spacers is that these molecules are more complex than the term "bifunctional" implies. Thus, in addition to antibodies against the azobenzenearsonate determinant, Ac-RAT-$(Pro)_{10}$-RAT raised anti-prolyl antibodies (Table I), demonstrating that the spacer also served as a complete or partial haptenic determinant. However, the conclusion that self-help applies in this situation appears to be justified since Ac-$(Pro)_{10}$-RAT did not provoke anti-RAT antibody (Table I), the second RAT moiety being essential for this response.

Earlier failure to induce humoral immunity with Ac-RAT-SAC-RAT (3) was hypothesized to be due to either intramolecular interaction between the two

RAT groups of the molecule, compromising its bifunctional character, or to the flexible spacer permitting both RAT determinants to interact with receptors on the same cell surface, blocking the requirement for cooperation between thymus-derived and bone marrow-derived lymphocytes. That intramolecular interaction did, in fact, occur was indicated by a hypochromic effect manifested by the compound. Success with Ac-Rat-$(Pro)_{10}$-RAT and Ac-TAT-$(Pro)_{10}$-TAT could not unambiguously discriminate between the two mechanisms since the rigid spacer, which effectively prevents intramolecular stacking as shown by the absence of hypochromism, might also prevent interaction of the two determinants with the same cell. However, the ability of Ac-TAT-SAC-TAT to evoke a humoral anti-TAT response (Table II) permitted a definitive discrimination between the alternatives. This compound carries the same flexible spacer as inactive Ac-RAT-SAC-RAT, but the TAT determinant has only electropositive (basic) groups in the physiologic pH range, so there is no tendency for intramolecular interaction to take place. Consequently, it may be concluded that self-help is biologically feasible, provided the determinants are prevented from associating with each other.

SUMMARY

L-Tyrosine-*p*-azobenzenearsonate (RAT) induces cellular immunity without humoral antibody in guinea pigs. Asymmetric bifunctional antigens composed of one RAT moiety and one dinitrophenyl (DNP) group separated by flexible spacers induce anti-RAT cellular immunity and an anti-DNP humoral response. Symmetrical bifunctional antigens of similar design but comprised of two RAT determinants induce cellular immunity without demonstrable anti-RAT antibody. However, when the flexible spacer is replaced by a rigid decaproline chain, humoral anti-RAT responses are provoked.

Since RAT contains both electropositive (azo) and electronegative (arsonate) centers, the failure of bifunctional RAT compounds with flexible spacers to induce humoral immunity might be ascribed either to intramolecular stacking, which compromises their bifunctional character, or to interaction of both determinants with receptors on the same cell surface, which would fail to satisfy the requirement for cooperation. In order to distinguish between these alternatives, symmetrical bifunctional antigens composed of two L-tyrosine-*p*-azophenyltrimethylammonium (TAT) determinants separated by flexible or rigid spacers were synthesized. TAT is immunogenic and does not cross-react with RAT. Furthermore, it contains only electropositive centers and consequently bifunctional molecules do not undergo intramolecular stacking. Immunization with either flexibly or rigidly spaced bifunctional TAT antigens raised anti-TAT antibody. These results conclusively demonstrate that "self-help," cooperation between bone marrow-derived and thymus-derived lymphocytes of identical or similar specificity, can occur, provided the determinants on the antigen are prevented from associating with each other.

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