ANTIGEN RECOGNITION AND THE IMMUNE RESPONSE

Structural Requirements in the Side Chain of Tyrosine for Immunogenicity of L-Tyrosine-Azobenzenearsonate*

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(Received for publication 25 May 1972)

Pursuant to the original observation that L-tyrosine-azobenzenearsonate (RAT, Table I) induced specific delayed hypersensitivity in guinea pigs (1), we demonstrated that this small molecule could serve as a carrier for macromolecular (2) or micromolecular (3) haptens. Indeed, the activity of RAT parallels that of conventional macromolecular immunogens. In addition to the induction of delayed cutaneous sensitivity, it triggered the release of migration-inhibitory factor and a proliferative response by cells from sensitized animals, and it prepared guinea pigs for secondary anti-hapten responses (2, 3).

An investigation of the structural requirements for immunogenicity of RAT revealed that other charged moieties could substitute for arsonate, including trimethylammonium which has an opposite charge. Modifications at the arsonate position yielded immunogens with distinctive specificities (3). Preliminary findings with compounds modified at the tyrosine position indicated that one, but not both, charged groups were essential for immunogenicity (3).

We now present findings with a complete series of analogs of RAT modified at the tyrosine position, which have been used to explore the importance of charge and the size of hydrophilic and hydrophobic side chains for the immunogenicity of this molecule.

Materials and Methods

Antigens.—*N-t*-Butyloxycarbonyl-L-tyrosine was purchased from Bachem Co., Marina Del Ray, Calif.; *p*-hydroxyphenylacetic acid, 3-(*p*-hydroxyphenyl)-propionic acid, tyramine, 4-*N*-propylphenol, 4-ethylphenol, and *p*-cresol were purchased from Aldrich Chemical Co., Milwaukee, Wis.; *p*-hydroxybenzoic acid was obtained from Eastman Kodak Co., Rochester, N. Y.; and *p*-hydroxyphenyl methyl carbinol was purchased from K & K Laboratories, Inc., Plainview, N. Y. These reagents were analyzed by thin-layer chromatography on silica gel and used without further purification. *p*-Hydroxyphenylethyl alcohol (tyrosol) was purchased from Research Organic Chemicals Co., Sun Valley, Calif., and purified by passage through a column of silica gel in ethyl acetate, followed by recrystallization in chloroform.

* Aided by U. S. Public Health Service Grants AI 05664, AM 08527, Training Grant AI 00299; and National Science Foundation Grant GB 27591.

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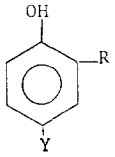
N-Acetyl-tyramine was obtained in poor yield by coupling acetic acid and tyramine using *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide. This procedure was necessary because direct acylation leads to an *N,O*-diacetyl product.

The phenolic compounds, with the exception of tyramine, were directly coupled with diazotized *p*-arsanilic acid. The products were invariably mixtures of mono- (orange) and

TABLE I

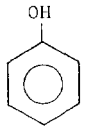
Synthetic Compounds Used as Antigens

Core compound:



Series of compounds with variations at position Y as follows:

-N = N-ϕ-p-AsO_3H_2= R



= A

Y	
$-CH_2-CHNH_2-COOH$	= T
$-CH_2-CH_2-COOH$	= O
$-CH_2-COOH$	= A
$-COOH$	= B
$-CH_2-CH_2NH_2$	= M
$-CH_2-CH_2-CH_3$	= N
$-CH_2-CH_3$	= E
$-CH_3$	= P
$-CH_2-CH_2OH$	= L
$-CH_2OH$	= H

bis- (purple) substituted phenols which were resolved by chromatography on Sephadex G-15 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in dilute ammonia. Tyramine was derivatized by diazonium coupling of *N-t*-butyloxycarbonyl tyramine, followed by purification on Sephadex G-15 and removal of the amino protective group by formic acid. The purity of all the diazotized products was investigated by thin-layer chromatography in a variety of solvents and by high-voltage electrophoresis at several pH values. The most useful solvent for investigating the purity of the azo compounds proved to be chloroform:methanol:concentrated ammonia (3:4:2).

Conjugates of several of the diazonium compounds with bovine serum albumin (BSA) were prepared as described previously (4, 5). The extent of substitution in each product was determined spectrophotometrically, using extinction coefficients obtained from the corresponding tyrosine derivatives. The average substitution varied from 3 to 11 chromophores per molecule of BSA.

Animals.—Randomly bred albino 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 equal volumes of complete Freund's adjuvant (CFA) (Difco Labs., Inc., Detroit, Mich.). Animals received 0.1 ml of the emulsion in each footpad.

Delayed Hypersensitivity.—Skin tests of animals were performed 21 days after immunization as previously described (3).

TABLE II

Induction of Delayed Hypersensitivity in Guinea Pigs by Analogs of RAT Modified at the Tyrosine Position

Test antigens	Immunizing agents											
	RAT	RAO	RAA	RAB	RAM	Ac-RAM*	RAN	RAE	RAP	RAL	RAH	CFA
$(RAT)_{11}$-BSA	17‡ (3)§	12 (7)	13 (3)	6 (3)	15 (4)	7‖ (3)	2 (3)	3 (3)	0 (3)	9 (3)	6 (3)	0 (6)
$(RAO)_4$-BSA	15 (3)	15 (7)	15 (3)	2 (3)	20 (4)	9‖ (3)	3 (3)	2 (3)	0 (3)	8 (3)	2 (3)	2 (6)
$(RAM\text{-}SAC)_{3}$-BSA	18 (6)	10 (7)	15 (3)	2 (3)	17 (4)	8‖ (3)	0 (3)	0 (3)	0 (3)	7 (3)	0 (3)	0 (6)
BSA	0 (3)	0 (7)	0 (3)	0 (3)	0 (4)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (3)	0 (6)

* The amino group of RAM is acetylated.
‡ Average delayed skin reaction in millimeters, read at 24 hr.
§ Number of animals tested.
‖ Very little induration.

RESULTS

Immunization of guinea pigs with the series of compounds listed in Table I resulted in clearly defined patterns of response which correlated with structure. The results of skin tests are given in Table II. Animals were skin tested with BSA conjugates of RAT, RAO, and RAM. Assays with each of these conjugates within each immunized group gave comparable results, indicating that specificity was directed largely or exclusively against the azophenyl-arsonate moiety. This was consistent with earlier findings which showed that substitution of other groups for arsonate, without altering the tyrosine moiety, yielded immunogens with distinctive specificities (3).

Molecules from which the amino group of the tyrosine side chain had been deleted (RAO, RAA, and RAB) retained immunogenicity, but a marked influence of chain length was evident. RAO and RAA, with 3-carbon and 2-carbon side chains, respectively, induced delayed hypersensitivity comparable with that given by the parent compound RAT; whereas RAB, with a single carbon

side chain, was at best marginally immunogenic under the conditions used for immunization. RAM, which is decarboxylated RAT (tyrosine substituted by tyramine), induced strong cellular immunity, whereas *N*-acetyl-RAM was much weaker, demonstrating the importance of charge as well as chain length for immunopotency.

Compounds containing hydrocarbon side chains, RAN, RAE, and RAP, did not induce significant cellular immunity regardless of side chain length. The requirement for a charged group was further assessed by testing compounds with side chains containing a polar hydroxyl group. RAL, with an ethanolic side chain, was comparable with Ac-RAM, and RAH, with a methanolic side chain, was weaker and closely paralleled the activity of RAB, the analog with a carboxylic side chain. However, based on a comparison of RAA and RAL, the carboxymethyl and ethanolic side chain compounds, respectively, the charged group appears to confer stronger immunogenicity than the polar group.

Guinea pigs injected only with CFA did not react to any of the test antigens and none of the animals reacted with unconjugated BSA.

DISCUSSION

RAT is probably the smallest molecule which has been shown to be immunogenic and, as such, offers an excellent model for study of the immune response at molecular and cellular levels. It has been shown that RAT can serve as a carrier for haptens (2, 3), and bifunctional antigen molecules have been fabricated to explore various molecular parameters of the immune response such as minimum essential spacing of determinants and the capacity to induce "self-help": the cooperation of an immunogenic determinant in the humoral response to a second identical determinant (3). In addition, the antihapten antibodies produced in response to small bifunctional antigens appear to be substantially less heterogeneous by isoelectric focusing than antibodies provoked by conventional hapten-protein conjugates (Roelants, G. E., and J. W. Goodman, unpublished observations), suggesting that RAT may have general utility as a carrier for the preparation of relatively homogeneous anti-hapten antibody of virtually any specificity.

In view of the utility of such a tool, it is of obvious importance to delineate the molecular parameters of RAT which are essential for immunogenicity, and to determine whether RAT is truly an independent immunogen or gives rise to cellular immunity via an indirect pathway, such as transconjugation or noncovalent association with macromolecules. A number of persuasive arguments for the intrinsic immunogenicity of RAT have previously been expounded (2, 3), including (*a*) the distinction between L and D isomers of tyrosine in conferring immunogenicity; (*b*) the ability of monofunctional RAT to provoke a pure cellular immune response, whereas azophenylarsonate-substituted proteins elicit anti-arsonate antibody without cellular immunity directed against the conjugated substituent; (*c*) the capacity of bifunctional hapten-RAT com-

pounds to raise anti-hapten antibody and cellular immunity directed against RAT; (*d*) the ability of RAT to prepare animals for secondary anti-hapten responses (carrier effect). To these may now be added the present findings that substitution of a hydrocarbon chain for the side chain of tyrosine abrogates the immunogenicity of the molecule. This would appear to preclude the possibility of immunogenicity as a consequence of the noncovalent association of the strongly anionic arsonate group with macromolecules. Indeed, it was shown earlier that arsonate itself is not essential for immunogenicity, but the substituent at that position determines specificity (3).

SUMMARY

The low molecular weight compound L-tyrosine-azobenzenearsonate (RAT) induces a cellular immune response in guinea pigs. The contribution of the side chain of tyrosine to the immunogenicity of RAT and the structural requirements at that position for immunogenicity were assessed by synthesizing a series of analogs of RAT containing modifications in the side chain of tyrosine and employing them as immunogens. Removal of either the carboxyl or amino group did not markedly affect immunogenicity, measured by the induction of delayed cutaneous sensitivity, whereas deletion of both completely abolished it. However, a charged group was not required since side chains containing a polar hydroxyl group could substitute for chains bearing an amino or carboxyl group. The size of the side chain exerted a pronounced influence; the charged or polar substituent had to be extended from the phenolic ring by at least two carbon atoms in order to confer immunogenicity.

We gratefully acknowledge the skilled technical assistance of Miss Inge M. Stoltenberg.

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