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Hapten-specific Response to a New Arsonic Acid Conjugate, Arsanil Isothiocyanate[1] (39654)

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The discovery of the azobenzenearsonate (ABA) hapten system has greatly facilitated the study of delayed hypersensitivity by providing an immunodominant haptenic group toward which this type of immunity can be studied in a similar way to what has been done in the study of humoral immunity. In general, specificity for this immunodominant group has been studied by skin testing (1) and *in vitro* reactions correlating with delayed hypersensitivity, i.e., blast transformation of lymphocytes from sensitized animals on exposure to antigen and inhibition of macrophage migration in the presence of these lymphocytes and antigen (2, 3).

It was initially found that delayed hypersensitivity was directed mainly to the ABA group with an indication that there was a minor contribution by the aromatic ring of the amino acid carrier (4). Further experiments indicated that structural changes in the α-amino acid portion of the tyrosine residue produced considerable changes in immunogenicity and that the presence of the *N*-acetyl and carboxyl groups in ABA-*N*-acetyltyrosine (ABA-tyr) played a major role in the immunogenicity of the conjugates as well as in their ability to induce *in vitro* blast transformation of specifically sensitized lymph node cells (5). Other workers have demonstrated, with the use of defined tetrapeptides containing *p*-azobenzenearsonate-L-tyrosine, that immunocompetent cells are able to differentiate between these compounds, usually responding best to the tetrapeptide employed to initiate the immune response (6).

In this study, a new conjugate of arsanilic acid, the arsanil isothiocyanate (Ars-NCS) determinant, was tested. This determinant couples via an isothiocyanate group to form a thiourea link to the ϵ-amino group of lysine. The capability of this new material to sensitize guinea pigs and to elicit delayed hypersensitivity was studied as well as its ability to produce cross-reactions in animals sensitized to the ABA groups.

Materials and methods. Azobenzenearsonate (ABA). ABA conjugates were made by coupling the appropriate amounts of the diazonium fluoborate salt of arsanilic acid to the carrier as described previously (7).

Arsanilic isothiocyanate (Ars-NCS). Ars-NCS conjugates were made from *p*-arsanilic acid isothiocyanate synthesized and kindly provided by Dr. A. Soloway (Dey, A., and Soloway, A., in preparation). Ars-NCS was added gradually to the carrier protein dissolved in 0.1 *M* phosphate buffer, pH 8.5, until a molar ratio of 100:1 was reached. The mixture was stirred at room temperature for 18–20 hr, maintaining the pH at about 8.5. In the case of conjugates of bovine serum albumin (Ars-NCS-BSA), purification was effected by precipitation with trichloroacetic acid and resolubilization with 0.01 *N* NaOH, followed by dialysis against a 0.05 *M* phosphate buffer at pH 7.0. Conjugates of egg albumin (Ars-NCS-EA) were dialyzed directly without precipitation. Since isothiocyanate reacts only with free amino groups, a ninhydrin assay was done on the conjugates to see how many NH_2 groups were left uncoupled. Then, assuming approximately 60 free NH_2 groups per mole of BSA, the number reacting with Ars-NCS was calculated by difference and found to be between 7–15 groups per molecule for the various preparations studied. Since no difference in skin reactivity was seen at this level of conjugation they were all used without further distinction.

Ars-NCS conjugate of ϵ-amino caproic acid (Ars-NCS-EACA). This was made by dissolving 262 mg (2×10^{-3} moles) of ϵ-amino caproic acid in 20 ml of 0.2 *M* borate buffer (pH 9) and adding, with stirring, 259

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mg (1×10^{-3} mole) of Ars-NCS. The pH was adjusted to 9 with NaOH, and stirring was continued over night at room temperature.

The solution was acidified with HCl and cooled in ice; the resulting precipitate was recovered by filtration and recrystallized from boiling water. The resulting solid, soluble in acetone, was used without further repurification.

Animals. Randomly bred albino female guinea pigs weighing 300–400 g were used.

Immunization. Two groups of guinea pigs were used. One was immunized with 2×10^{-6} mole of ABA-tyr per animal contained in a 0.1-ml emulsion made with Freund's complete adjuvant (8.5 parts mineral oil, 1.5 parts arlacel-A, and 5 mg of killed tubercle bacilli/ml), and distributed among the four footpads. The other group received a similar emulsion containing Ars-NCS-EACA at a dose of 2×10^{-6} mole per guinea pig.

Skin tests. Three weeks after immunization, the animals were shaved, depilated, and skin tested with 30 μg each of ABA-BSA, ABA-EA, Ars-NCS-BSA, Ars-NCS-EA, and old tuberculin (OT, 1:400) in saline. Animals used for *in vitro* experiments were skin tested with 1 μg of the ABA and Ars-NCS compounds prior to sacrifice to determine reactivity.

The sites of skin tests were observed 3 hr later for the presence of Arthus reaction, if any, and, subsequently, at 24 hr for measurement of the average diameter and degree of induration of the reaction.

In vitro stimulation of guinea pig lymphocytes. The guinea pigs were anesthetized with ether and bled to death by cardiac puncture. The popliteal, inguinal, axillary, and subscapular lymph nodes were removed using a sterile procedure. The pericapsular fat was dissected free, and the lymph nodes were teased with forceps in petri dishes containing culture medium kept cold. The cell suspension was passed through a sterile wire screen, and the cells were washed and resuspended in culture medium. The culture medium used was RPMI 1640 containing penicillin (100 units/ml) and streptomycin (50 mg/ml; Associated Biomedic Systems, Inc., Buffalo, N.Y.). Added were Kanamycin (2.5 mg/100 ml), 200 m*M* L-glutamine (0.5 ml/100 ml), and 10% heat-inactivated guinea pig serum (Grand Island Biological Co., Grand Island, N.Y.). Viable cell counts were made by the trypan blue dye exclusion method. Cell suspensions of 2×10^6 cells/ml were prepared, and 2-ml cultures were set up in triplicate in 12 × 75-mm sterilized glass tubes with loosely fitting aluminium caps. Antigens were added in 0.1-ml volumes to a final concentration of 12.5, 25, 50, 100, and 200 μg/ml. The cells were then cultured for a total of 72 hr in a 37° humidified incubator with an atmosphere of 5% CO_2 in air. Forty-eight hours after setting up the cultures, 1 μCi of [^{3}H]thymidine (Schwarz/Mann, Orangeburg, N.Y., Specific activity: 3 Ci/mole) was added to each culture tube in a volume of 0.1 ml. The cultures were then harvested by washing once with phosphate-buffered saline, pH 7.4, at room temperature and twice with 10% trichloracetic acid (TCA) in the cold. A drop of 20% serum in saline was added as a carrier before adding TCA for the first time. The final precipitates were dissolved in 0.5 ml of Soluene-100 (Packard Instrument Co., Downers Grove, Ill.) per tube. These were transferred to glass vials containing 10 ml of scintillation fluid (16 g of PPO and 0.4 g of POPOP in 3.8 liter of toluene). The tubes were washed twice with 1 ml of the scintillation fluid which was added to the vials. The radioactivity was measured in a Packard liquid scintillation counter, and the counts per minute were recorded. The results were analyzed statistically by the Student's *t*-test, and differences which had a P value less than 0.05 were considered significant.

Results. In the first experiment, three guinea pigs were immunized with ABA-tyr and three with Ars-NCS-EACA in CFA. The skin tests were performed at 3 weeks with 30 μg of ABA-BSA and Ars-NCS-BSA and with OT 1:400. The results of the skin tests at 24 hr are presented in Table I.

It was seen that Ars-NCS-EACA was an immunogen capable of producing hapten-specific delayed hypersensitivity as all the animals gave strong delayed reactions averaging 18 mm of induration and erythema with Ars-NCS-BSA. There was a remarka-

ble degree of cross-reaction with ABA-BSA in these animals, the mean diameter of the reactions being 15 mm of induration and erythema.

The animals immunized with ABA-tyr showed, as previously, a positive reaction to ABA-BSA with a mean diameter of 16 mm of marked erythema and induration in all the animals as well as a central blanche in two of them. These animals also gave reactions averaging 13 mm of erythema and induration with the Ars-NCS-BSA. Thus, in both cases, cross-reaction was evident although the homologous conjugates elicited a somewhat more intense skin test. There were no Arthus reactions observed at 3 hr in any of these six animals. All reactions to OT were of comparable intensity.

The experiment was repeated with six more animals, and, this time, they were skin tested with 30 μg of egg albumin conjugates ABA-EA and Ars-NCS-EA. The results shown in Table II demonstrated essetially the same capability of Ars-NCS-EACA to function as an immunogen for hapten-specific delayed hypersensitivity. Three animals immunized with Ars-NCS-EACA responded to Ars-NCS-EA with a skin test having a mean diameter of 18 mm. The response to ABA-EA was approximately 8 mm with somewhat less induration. A cross-reaction was also observed in the case of animals immunized with ABA-tyr, the mean reaction observed with ABA-EA being 17 mm and with ABA NCS-EA, 15 mm. Again, in both instances, reactions to the homologous conjugates were somewhat larger and more intense.

The ability of lymphocytes from animals immunized with Ars-NCS-EACA to undergo *in vitro* stimulation by antigen was tested next. In the outbred population of guinea pigs used in this study, the response in terms of skin reaction with both type of conjugates were variable from animal to animal, but skin reactions were generally paralleled by *in vitro* responses to the same antigen. Therefore, animals which had a poor skin reaction to a test dose of 1 μg of the homologous conjugate and did not show significant stimulation *in vitro* are not presented in the study.

TABLE I. DELAYED REACTIONS TO ABA AND ARS-NCS CONJUGATES OF BSA IN GUINEA PIGS IMMUNIZED WITH ABA-TYR OR ARS-NCS-EACA IN COMPLETE FREUND'S ADJUVANT.

Guinea pig number	Immunized with	Delayed reactions to ABA-BSA (30 μg)		Ars-NCS-BSA (30 μg)		OT	(1:400)
1	ABA-tyr	14[a]	++[b]	7	±	12	++
2	ABA-tyr	17	+++	16	++	16	+++
3	ABA-tyr	17	+++	15	++	12	++
4	Ars-NCS-EACA	12	++	20	++	18	+++
5	Ars-NCS-EACA	20	++++	17	++	22	++++
6	Ars-NCS-EACA	14	++	17	++	12	++

[a] Diameter of erythema in millimeters.
[b] Degree of induration.

TABLE II. DELAYED REACTIONS TO ABA AND ARS-NCS CONJUGATES OF EGG ALBUMIN IN GUINEA PIGS IMMUNIZED WITH ABA-TYR OR ARS-NCS-EACA IN COMPLETE FREUND'S ADJUVANT.

Guinea pig number	Immunized with	Delayed reactions to ABA-Ea (30 μg)		Ars-NCS-Ea (30 μg)		OT	(1:400)
1	ABA-tyr	16[a]	++[b]	12	++	12	++
2	ABA-tyr	18	+++	17	+++	14	++
3	ABA-tyr	18	+++	15	++	14	+++
4	Ars-NCS-EACA	11	++	18	+++	16	+++
5	Ars-NCS-EACA	4	+	20	++++	18	+++
6	Ars-NCS-EACA	11	++	17	+++	13	+++

[a] Diameter of erythema in millimeters.
[b] Degree of induration.

Typical dose-response curves to monomeric conjugates (Ars-NCS-EACA and ABA-tyr) are seen for an animal immunized with Ars-NCS-EACA (Fig. 1a) and an animal immunized with ABA-tyr (Fig. 1b). In both instances, either monomeric antigen produced significant degrees of stimulation, but it was apparent that the homologous conjugate used for immunization produced better stimulation *in vitro*. When similar responses to the polyvalent conjugates (Ars-NCS-EA and ABA-EA) were studied in a guinea pig immunized with Ars-NCS-EACA (Fig. 2a) or with ABA-tyr (Fig. 2b), it was again apparent that a large degree of cross-reactivity was present, in that both conjugates produced good responses with both sets of lymphocytes. This time, however, less difference was seen between the homologous and heterologous conjugate.

In an effort to determine whether the specificity or degree of cross-reaction between Ars-NCS and ABA conjugates changed with time, groups of guinea pigs immunized with either Ars-NCS-EACA or ABA-tyr were sacrificed at intervals up to 12 weeks, and their lymphocytes were cultured *in vitro* with the various conjugates to measure [^{3}H]thymidine incorporation.

The results shown in Table III and IV are the means of triplicate samples at the particular dose which showed maximum stimulation for that particular antigen (usually 100 or 200 μg/ml). In virtually all cases, stimulation with the homologous monomeric conjugate was best and a comparison of homologous conjugate stimulation to heterologous conjugate stimulation gave ratios averaging 12:1 (range 2–42) for both sets of immunized pigs. Stimulation measured with the polyvalent conjugates did not show the same consistent trend toward favoring the homologous conjugate, and ratios of stimulation of homologous to heterologous polyvalent conjugates in both groups of pigs were closer to 1:1 (range 0.17–6.7).

There was no clear cut change in stimulability with time either in the degree of stimulation by the homologous conjugate or in the nature of the cross-reactivity between the two types of conjugate.

Discussion. Guinea pigs immunized with Ars-NCS-EACA in complete Freund's adjuvant became sensitized for a delayed hy-

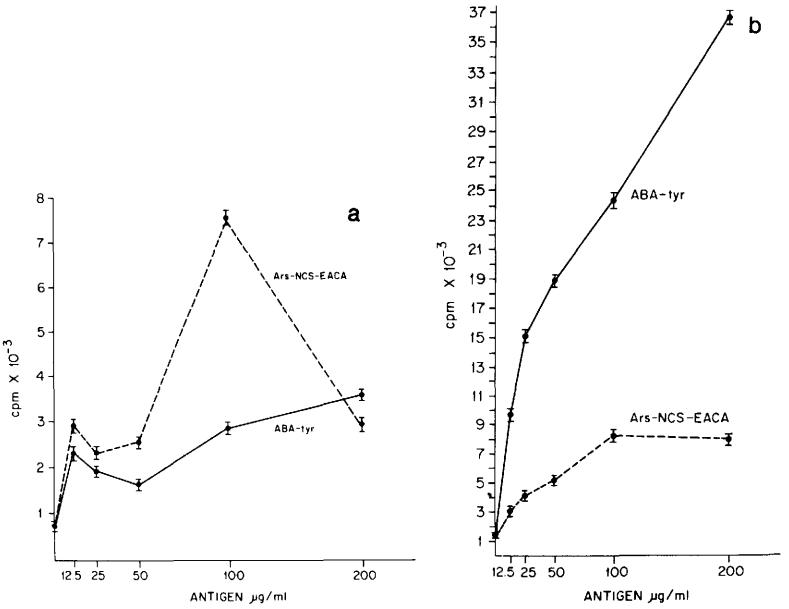

FIG. 1. Typical dose-response curve to Ars-NCS-EACA and ABA-tyrosine *in vitro* by cells from a guinea pig immunized with Ars-NCS-EACA (a) and ABA-tyr (b). the results are expressed as the (mean of the counts/minute $\times$ 10^{-3}) $\pm$ the standard error of the replicate samples. The dose of antigen is expressed in micrograms/milliliter of culture medium.

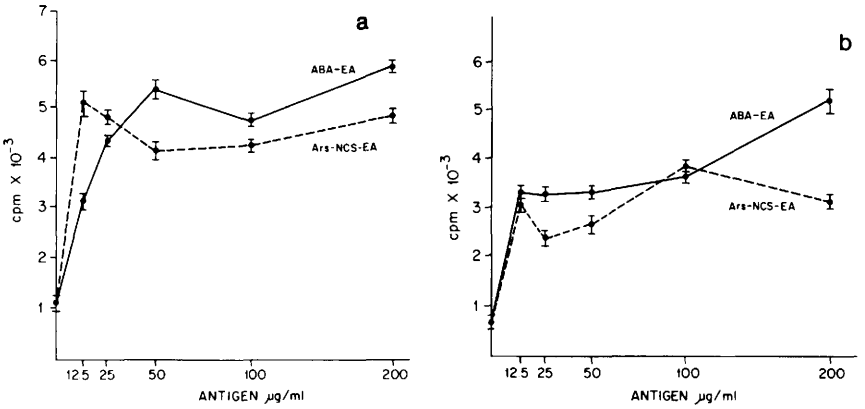

FIG. 2. Typical dose-response curve to ABA-EA and Ars-NCS-EA *in vitro* by cells from a guinea pig immunized with Ars-NCS-EACA (a) and ABA-tyr (2b). The results are expressed as the (mean of the counts/minute $\times 10^{-3}$) $\pm$ the standard error of the replicate sample. The dose of antigen is expressed in micrograms/milliliter of culture medium.

TABLE III. ANTIGEN-INDUCED [^{3}H]THYMIDINE INCORPORATION BY LYMPHOCYTES FROM GUINEA PIGS IMMUNIZED WITH ARS-NCS-EACA AND STIMULATED WITH ABA AND ARS-NCS CONJUGATES.[a]

Guinea pig number	Time of testing (weeks)	Immunized with	Antigen used *in Vitro*	Radioactivity (mean cpm of test − mean cpm of control)	Ratio of homologous:heterologous conjugate stimulation	
					Monovalent	Multivalent
1	4	Ars-NCS-EACA	ABA-Tyr	268 (N.S.)[b]	38.2	
			Ars-NCS-EACA	10,229		
			ABA-BSA	11,833		0.19
			Ars-NCS-BSA	2,278		
2	5	Ars-NCS-EACA	ABA-Tyr	768	6.2	
			Ars-NCS-EACA	4,733		
			ABA-EA	4,758		0.85
			Ars-NCS-EA	4,060		
3	5	Ars-NCS-EACA	ABA-Tyr	2,233	8.0	
			Ars-NCS-EACA	17,932		
			ABA-EA	635		6.3
			Ars-NCS-EA	4,029		
4	7	Ars-NCS-EACA	ABA-Tyr	2,595	2.5	
			Ars-NCS-EACA	6,606		
			ABA-BSA	1,575		5.0
			Ars-NCS-BSA	7,955		
5	12	Ars-NCS-EACA	ABA-Tyr	2,937	7.7	
			Ars-NCS-EACA	22,582		
			ABA-BSA	441 (N.S.)		5.9
			Ars-NCS-BSA	2,645[c]		
					Average 12.5	3.6

[a] The result for each antigen is the mean of triplicate samples at the dose of antigen which gave the maximum incorporation.

[b] N.S., not significant.

[c] P value of <0.05. All other differences have a value <0.01.

persensitivity which could be elicited with other arsanil conjugates. These conjugates were of two types, those in which the arsanilic group was coupled via an NCS linkage to lysine and others, in which the diazo linkage to tyrosine and histidine was present. Marked cross-reactivity in the *in vivo* response was observed. For example, a group of animals immunized with Ars-NCS-EACA gave an average reaction of 18 mm

TABLE IV. ANTIGEN-INDUCED [^{3}H]THYMIDINE INCORPORATION BY LYMPHOCYTES FROM GUINEA PIGS IMMUNIZED WITH ABA-TYROSINE AND STIMULATED WITH ABA AND ARS-NCS CONJUGATES.[a]

Guinea pig number	Time of testing (weeks)	Immunized with	Antigen used *in Vitro*	Radioactivity (mean cpm of test − mean cpm of control)	Ratio of homologous:heterologous conjugate stimulation	
					Monovalent	Multivalent
1	4	ABA-Tyr	ABA-Tyr	35,396	5.1	
			Ars-NCS-EACA	6,877		
			ABA-BSA	18,787		
			Ars-NCS-BSA	−364 (N.S.)[b]		
2	5	ABA-Tyr	ABA-Tyr	2,289	8.8	
			Ars-NCS-EACA	258		
			ABA-EA	4,409		1.4
			Ars-NCS-EA	3,062		
3	6	ABA-Tyr	ABA-Tyr	40,898	3.0	
			Ars-NCS-EACA	13,393		
			ABA-BSA	8,337		1.1
			Ars-NCS-BSA	7,474		
4	9	ABA-Tyr	ABA-Tyr	15,681	42.0	
			Ars-NCS-EACA	372		
			ABA-EA	3,114		2.0
			Ars-NCS-EA	1,485		
5	12	ABA-Tyr	ABA-Tyr	47,253	2.0	
			Ars-NCS-EACA	23,569		
			ABA-BSA	677(N.S.)		.17
			Ars-NCS-BSA	3,903		
					Average 12.1	1.1

[a] The result for each antigen is the mean of triplicate samples at the dose of antigen which gave the maximum incorporation.

[b] N.S., not significant; all other differences have a P value of >0.01.

(diameter) with Ars-NCS-BSA and 15 mm (diameter) with ABA-BSA. All these responses were accompanied with marked erythema and induration. A similar cross-reaction was seen on skin testing animals which had been immunized with ABA-tyr in complete Freund's adjuvant. A group of such animals responded with an average skin test diameter of 17 mm to ABA-NCS-EA. Again, all these reactions were accompanied by marked erythema and induration.

In vitro stimulation of lymph node lymphocytes taken from animals immunized with Ars-NCS-EACA and ABA-tyr was also successful with antigens which included the monovalent compounds as well as the multivalent forms, ABA-BSA, ABA-EA, Ars-NCS-BSA, and Ars-NCS-EA, and, essentially similar cross-reactivity was seen, thus, further demonstrating the immunodominance of the arsonic acid group (4).

In vitro reactions tended to indicate a finer degree of discrimination than did *in vivo* reactions, especially when monovalent conjugates were used as the test antigens. Thus, lymphocytes from animals immunized with either ABA-tyr or Ars-NCS-EACA gave greater degrees of stimulation with the homologous conjugates. The average ratio of homologous to heterologous conjugate stimulation was about 12:1 in both instances (Table III and IV). However, these differences tended to disappear when multivalent conjugates were used, and the average ratios of homologous to heterologous conjugate stimulation were closer to unity.

The greater stimulation produced by the homologous monovalent conjugates could be a reflection of the contribution by the NCS or diazo linkage groups, as well as the lysine or tyrosine attachment residues to the immunogenic units. Similar extensions of the immunoreactive grouping beyond the ABA moiety were observed in other studies of cell-mediated immunity (3, 6, 8). Therefore, for example, if only the arsonic acid group of Ars-NCS-EACA was able to fit the T-cell receptor for the ABA-tyr immunogenic unit, insufficient binding affinity to trigger the cell might be generated. How-

ever, the possibility of multipoint attachment which could be achieved by the polyvalent protein conjugates may make triggering of cross-reactions more likely and, thus, may offer an explanation for smaller differences in reactivity of the multivalent compounds.

Reactivity, in terms of both skin tests and *in vitro* stimulation, was seen in animals up to 12 weeks (which was the longest duration studied). Some studies in rats and guinea pigs with ABA-tyr, ABA-histidine, and ABA-trypotophan (9, 10) have indicated that the fine specificity recognition beyond the ABA group (as tested by inhibition of macrophage migration in the presence of sensitized lymphocytes and antigen) is not seen as early as 3–4 weeks after immunization, but develops later. No such increase in sensitivity or 'immunological maturation' was observed in the present study: in fact, the fine specificity recognition was already present as early as 4 weeks. It is possible that examination of more animals over a longer period of time might provide some evidence of a "maturation" of the immune response, but the present data are insufficient to arrive at any conclusion other than the high degree of arsonic acid specificity seen with both types of conjugate.

Summary. A new derivative of arsonic acid, arsanil isothiocyanate was used to couple arsonic acid via an isothiocyanate group to amino groups. Immunization of guinea pigs with this determinant attached to ϵ-amino carproic acid in complete Freund's adjuvant led to the production of delayed hypersensitivity toward this haptenic determinant, as indicated by positive skin tests to compounds to which the determinant was coupled to egg albumin and bovine serum albumin. In addition, lymphocytes from these immunized animals responded to these antigens *in vitro* by undergoing blast transformation as measured by incorporation of tritiated thymidine.

The dominance of the arsonic acid group in the determinant was seen in the form of positive skin tests as well as in *in vitro* stimulation with the various conjugates in animals immunized with either Ars-NCS-EACA or ABA-tyr.

The response by the homologous compounds were, however, generally better than those of the heterologous compounds. These results suggest an important contribution to the reactivity of the immunodominant arsonic acid group by the neighboring site of attachment.

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