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Specificity of murine delayed-type hypersensitivity to conjugates of large or small haptens on protein carriers bearing lipid groups

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Summary. Delayed-type hypersensitivity (DH) in the mouse was provoked with different hapten–carrier complexes mixed with the cationic, surface-active lipid, dimethyl dioctadecyl ammonium bromide (DDA). DH was measured as footpad swelling.

Conjugates of bovine serum albumin (BSA) with the small haptens dinitrophenyl (DNP), 'arsonate' (ARS) and 'sulphonate' (SULPH) served to generate strong DH reactions towards the homologous antigen. Insertion of a tripeptide spacer between the hapten and carrier resulted in lower DH reactivity. Optimal dosages and optimal time intervals between sensitization and DH elicitation were determined for the enlarged hapten–carrier complexes. Cyclophosphamide (CY) treatment, before priming with complexes mixed with DDA, caused a 5–6 day delay in the expression of DH but failed to evoke enhanced DH for any of the antigens tested.

A broad array of cross reactions between small and enlarged hapten–carrier complexes showed a relative lack of specificity in these DH responses. The results are compared with others reported in the literature and are explained mainly by the effects of electrostatically bound lipid groups of DDA in the sensitizing conjugates.

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INTRODUCTION

Immunization of guinea-pigs with the hapten 2,4-dinitrophenyl (DNP) coupled directly to mycobacteria generated T lymphocytes responsive to DNP on a wide variety of carriers (Janeway, Cohen, Ben Sasson & Paul, 1975). These results contrast with most T-cell responses to hapten–carrier conjugates which are specific for carrier or for specific conjugate determinants, but not for the same hapten on heterologous carriers (Paul, 1970). It was further demonstrated (Janeway, Maurer, Daily & Inman, 1976) that hapten-specific T cells precisely discriminate the hapten used for priming from other haptens. They also showed that insertion of a tripeptide spacer between the hapten and carrier interfered with recognition of the hapten. This suggested that the carrier amino acids make essential contributions to the determinants or epitopes recognized by T cells.

The capacity of hapten coupled mycobacteria to activate T cells to 'conjugate-specific' determinants was also found with DNP-BSA when lipid groups were covalently attached to the BSA (Daily & Hunter, 1974). They also used the cationic, surface-active lipid, dimethyl dioctadecyl ammonium bromide (DDA) to introduce lipid groups via electrostatic bonds with the hapten–carrier complexes. Immunization of guinea-pigs (Daily &

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Hunter, 1974) or mice (Snippe, Belder & Willers, 1977) with DNP–carrier complexes mixed with DDA produced hapten-specific delayed type hypersensitivity (DH). The purpose of the present study was to investigate the specificity of DH in mice immunized with complexes of different small or enlarged haptens on carriers bearing electrostatically-bound lipid groups of DDA.

MATERIALS AND METHODS

Animals and immunization

Inbred female BALB/c mice were obtained from the Animal Production Section, NIH, and were used at an age of about 10 weeks. For each experiment, groups containing six to eight mice were used. Different amounts of hapten–protein conjugate, dissolved in 0·1 ml saline and homogenized with 0·1 ml (100 μg) of DDA (Eastman Kodak, New York), were injected intracutaneously (i.c.) in the abdomen, at two separate sites.

Antigens

The haptens employed in this study are listed in Table 1. Large haptens have been given *single* capital letter designations. The abbreviations DNP, ARS and SULPH have been employed for the small haptens. The procedures for synthesis of the special tripeptide-enlarged haptens were those reported by Inman, Merchant & Tacey (1973). DNP-BSA coupling was carried out and analysed according to the method of Eisen, Carsten & Belman (1954). ARS-BSA and SULPH-BSA were prepared according to the method of Tabachnick & Sobotka (1960). Coupling of enlarged haptens to protein was performed as described by Inman, Merchant, Claflin & Tacey (1973). Haptenated Ficoll antigens were prepared as described by Inman (1975).

Assay for delayed-type hypersensitivity (DH)

The DH reactions were determined by measuring the increase in footpad thickness (footpad swelling test) as described by Kerckhaert, van den Berg & Willers (1974). In all the experiments the test dose of 10 μg of antigen suspended in 0·05 ml saline was injected into the left hind footpad. A footpad swelling of 0·25 mm is regarded as positive. The results are expressed as the increment of the footpad thickness in 0·1 mm units ± the standard error of the mean (SEM) for six to eight mice.

Cyclophosphamide treatment

Cyclophosphamide (CY) was obtained from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). The mice received an intraperitoneal (i.p.) injection of CY (300 mg/kg) in 0·5 ml of saline, 8 h before i.c. immunization with antigen.

RESULTS

Optimal dose for DH on enlarged hapten–carrier complexes

In previous experiments Snippe, Belder & Willers (1977) showed that the maximal footpad swelling

Table 1. Letter symbols and chemical designation* for haptens employed in this study

Small haptens
DNP = N-2,4-dinitrophenyl
ARS = p-azobenzenearsonate
SULF = p-azobenzenesulphonate

Large haptens

TGG series
A = 3-(p-arsonophenylazo)-N-acetyl-L-tyrosylglycylglycine
M = 3-(p-trimethylaminophenylazo)-N-acetyl-L-tyrosylglycylglycine
θ = 3-(phenylazo)-N-acetyl-L-tyrosylglycylglycine
V = 3-(p-carboxyphenylazo)-N-acetyl-L-tyrosylglycylglycine
G = unsubstituted N-acetyl-L-tyrosylglycylglycine

AGG series
K = N-(2,4,6-trinitrophenyl)-β-alanylglycylglycine
N = N-(4-hydroxy-3-iodo-5-nitrophenylacetyl)-β-alanylglycylglycine

* Structural formulae for most of the large haptens are presented in the reference of Inman, Merchant & Tacey (1973).

Table 2. Dose effect in delayed-type hypersensitivity

Antigen	Sensitization (μg)	Footpad swelling in 0·1 mm ± SEM: Elicitation (μg) 3	10	30
K_{23}-BSA	3	10·5 ± 1·1	10·3 ± 0·3	6·0 ± 1·1
	10	11·7 ± 1·2	12·6 ± 1·5	11·3 ± 0·6
	30	6·4 ± 0·8	9·3 ± 1·7	10·2 ± 1·2
	100	8·6 ± 1·3	6·3 ± 1·1	10·4 ± 1·7
J_{30}-BSA	3	6·5 ± 1·3	5·3 ± 0·9	
	10	6·2 ± 1·5	8·8 ± 1·6	
	30	5·1 ± 1·0	7·8 ± 0·5	
	100	6·6 ± 2·2	7·5 ± 1·2	
A_{22}-BSA	3	4·3 ± 0·6	5·0 ± 1·6	7·7 ± 1·3
	10	4·7 ± 1·5	12·8 ± 1·8	10·8 ± 1·2
	30	4·7 ± 1·2	9·5 ± 1·1	10·0 ± 2·0
	100	8·5 ± 1·5	8·7 ± 1·9	8·0 ± 2·2

Groups of five mice were immunized with 3, 10, 30 or 100 μg antigen in 100 μg DDA and 5 days later elicited with 3, 10 or 30 μg of the homologous antigen.

after priming with antigen in DDA was measured at days 5 and 6. With this time interval the optimal dose was determined for sensitization and elicitation of DH with the enlarged hapten–carrier complexes. Groups of five mice were immunized with 3, 10, 30 or 100 μg of K_{23}-BSA, J_{30}-BSA or A_{22}-BSA in 100 μg DDA and 5 days later were challenged with 3, 10 or 30 μg of the homologous antigen (Table 2). Optimal responses were obtained with 10 μg used both for priming and eliciting injections. Unexpectedly, the response to J_{30}-BSA was less than the response to K_{23}-BSA or to A_{22}-BSA. This phenomenon was not dose dependent (unpublished data).

Table 3. Delayed-type hypersensitivity reactions to hapten–carrier complexes

Small haptens	Footpad swelling in 0·1 mm ± SEM	Enlarged haptens	
DNP_5-BSA	3·0 ± 0·6	K_{23}-BSA	13·7 ± 1·0
DNP_{31}-BSA	18·4 ± 1·2	J_{30}-BSA	7·8 ± 0·4
ARS_7-BSA	14·2 ± 1·2	A_{22}-BSA	11·8 ± 1·3
$SULF_{16}$-BSA	13·6 ± 0·7	N_{43}-BSA	11·7 ± 0·9
ARS_7-OVA	14·4 ± 2·0	θ_{41}-BSA	8·4 ± 0·5
		G_{25}-BSA	2·3 ± 0·5
		M_{16}-BSA	1·8 ± 0·3
		θ_9-HGG	6·2 ± 0·4

Groups of mice (five to seventeen per group) were immunized i.c. with 10 μg of antigen in 100 μg of DDA and 5 days later responses were elicited with 10 μg of homologous antigen.

DH on small and enlarged hapten–carrier complexes

As shown earlier, DH was influenced by the hapten–carrier ratio in DNP-BSA complexes (Snippe, Willers, Grave & Kamp, 1975; Snippe *et al.*, 1977). To investigate the role of the hapten, groups of mice were immunized with 10 μg of a small or an enlarged hapten–carrier complex in DDA and, 5 days later, responses were elicited with 10 μg of the homologous antigen. A strong DH response was observed with the small hapten–carrier complexes DNP_{31}-BSA, ARS_7-BSA, $SULPH_{16}$-BSA and ARS_7-OVA (Table 3). The enlarged hapten complexes K_{23}-BSA, A_{22}-BSA and N_{43}-BSA were slightly less reactive, while J_{30}-BSA, θ_{41}-BSA and θ_9-HGG gave only moderate reactions. The small hapten complex DNP_5-BSA and the enlarged hapten complexes G_{25}-BSA and M_{16}-BSA gave no significant DH. Neither the injection of the thymus independent antigens J_{59}-Ficoll and J_{40}-Ficoll alone (at various doses) with DDA nor the simultaneous injection of these thymus independent antigens and BSA resulted in DH (data not given).

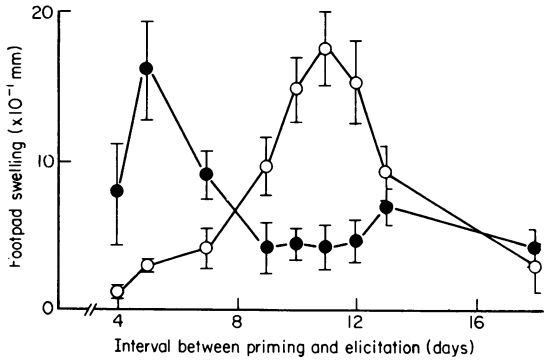

Figure 1. The effect of CY treatment before priming on DH. Groups of eight mice were immunized i.c. with 10 μg of K_{23}-BSA in DDA 8 h after i.p. injection of saline (●) or CY (○). Challenge with 10 μg of K_{23}-BSA was performed by footpad injection at varying time intervals thereafter. The increase in footpad thickness was measured 24 h after challenge. Vertical bars indicate SEM.

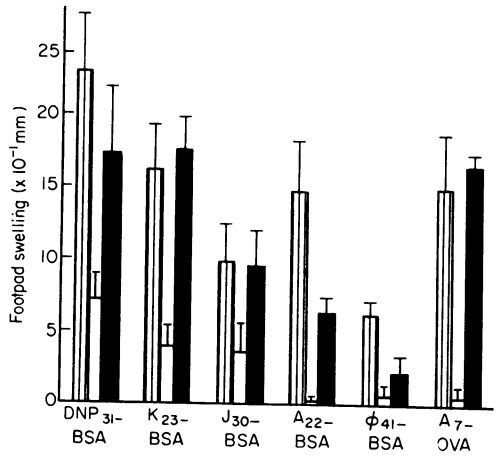

Figure 2. Homologous DH reactions with different hapten–carrier complexes. Groups of eight mice were immunized with 10 μg of antigen in DDA 8 h after i.p. injection of saline (▥, □) or CY (■). DH responses were elicited in these mice by left footpad injection of the same dose of the homologous antigen at day 5 (▥) or day 11 (□, ■). The increase in footpad thickness was measured 24 h later. Vertical bars indicate SEM.

Interval between priming and eliciting injections with K_{23}-BSA

In a previous paper (Snippe, Belder & Willers, 1977) the effect of the interval between priming and eliciting injections on DH to DNP_{28}-BSA was studied with and without pretreatment with CY. The present study was extended to the enlarged hapten–carrier complex, K_{23}-BSA. At different intervals after immunization with 10 μg of K_{23}-BSA, groups of six mice were elicited with 10 μg of K_{23}-BSA. In control mice DH was present at day 4, reached a maximum at day 5 and decreased slowly thereafter (Fig. 1). To investigate the effect of CY, groups of mice received an i.p. injection of CY (300 mg/kg) and were sensitized 8 h later with 10 μg of K_{23}-BSA. The DH response was markedly impaired until about 7 days after immunization but was fully expressed between days 9 and 12 (Fig. 1). No difference in the maximum height of the response (day 11 vs day 5) was observed.

The effect of CY treatment on DH to different hapten–carrier complexes

The influence of CY treatment on DH was further analysed for other hapten–carrier complexes. Groups of mice received CY 8 h before the antigen was given. DH responses were elicited with the homologous antigen at day 11. Control groups received no CY and their responses were elicited at day 5 or day 11. Fig. 2 shows that in all instances the response of the control groups is lower at day 11 than at day 5. In most instances, however, CY treatment resulted in a maximal response at day 11 which was closely comparable to the response of the controls at day 5. This delay and recovery of amplitude after CY treatment was not obtained to the same degree with A_{22}-BSA or with θ_{41}-BSA.

Cross reactions between small haptens

To determine the haptenic specificity of DH in mice, cross reactions between small haptens were studied. Groups of mice immunized with 10 μg of DNP_5-BSA, DNP_{31}-BSA, ARS_7-BSA and $SULPH_{16}$-BSA were challenged with the homologous or a heterologous antigen 5 days later (Table 4). Mice immunized with DNP_5-BSA were responsive only to DNP_{31}-BSA. Mice sensitized with DNP_{31}-BSA showed a strong response to the homologous antigen but also showed an appreciable response to $SULPH_{16}$-BSA and to ARS_7-BSA. Priming with ARS_7-BSA or $SULPH_{16}$-BSA resulted in cross reactions to $SULPH_{16}$-BSA and to ARS_7-BSA, respectively.

Table 4. Cross reactions in delayed-type hypersensitivity between small haptens on an identical carrier

	Footpad swelling in 0·1 mm ± SEM			
	Elicitation			
Sensitization	DNP_5-BSA	DNP_{31}-BSA	ARS_7-BSA	$SULF_{16}$-BSA
DNP_5-BSA	2·3 ± 0·4	9·1 ± 0·6*	3·5 ± 0·4†	2·7 ± 0·6‡
DNP_{31}-BSA	4·9 ± 0·8*	17·6 ± 0·9	7·9 ± 1·1*	10·7 ± 1·4*
ARS_7-BSA	1·9 ± 0·6*	3·6 ± 0·7*	13·2 ± 0·8	13·4 ± 0·9‡
$SULF_{16}$-BSA	2·9 ± 0·5*	4·9 ± 0·6*	12·2 ± 0·5*	14·9 ± 0·8

Groups of mice were immunized i.c. with 10 μg of antigen in 100 μg DDA, and 5 days later their DH responses were elicited with 10 μg of a test antigen. The following symbols denote *P* values obtained by comparing the effectiveness of the homologous antigen versus that of other antigens in eliciting DH among groups of mice, all of which received the same sensitizing antigen. * $P < 0.005$; † $P < 0.025$; ‡ *P* value not significant.

Cross reactions between enlarged haptens

Enlarged haptens generate multiple diverse populations of antibody forming cells (Merchant & Inman, 1977), so it is possible that this effect might also be reflected in DH responses to enlarged haptens. Groups of mice immunized with 10 μg of K_{23}-BSA, J_{30}-BSA, A_{22}-BSA or N_{43}-BSA received the standard eliciting injections on day 5. Mice sensitized with A_{22}-BSA or N_{43}-BSA showed a strong response to the homologous antigen and weaker reactions to most other antigens (Table 5). On the other hand, mice immunized with K_{23}-BSA showed a strong DH reaction to the homologous antigen and also to a number of other antigens including the non-related antigens A_{22}-BSA and N_{43}-BSA. Similar results were obtained after sensitization with J_{30}-BSA. Although the optimal DH response was generally lower, the cross reactions tended to occur with the same antigens as for K_{23}-BSA. When used for sensitization, the enlarged haptens A and N stimulated DH which was far more specific than that seen for J and K.

Cross reactions between small and enlarged haptens

In order to evaluate the capacity for haptenic recognition in the receptors on those cells expressing DH, the influence of a spacer between hapten and carrier was studied. Groups of mice were immunized with DNP_{31}-BSA, ARS_7-BSA or $SULPH_{16}$-BSA and their

Table 5. Cross-reactions in delayed-type hypersensitivity between large haptens on an identical carrier

	Footpad swelling in 0·1 mm ± SEM Elicitation								
Sensitization	BSA	K_{23}-BSA	J_{30}-BSA	A_{22}-BSA	N_{43}-BSA	θ_{41}-BSA	G_{25}-BSA	M_{16}-BSA	V_6-BSA
K_{23}-BSA	1·5 ± 0·3*	14·2 ± 0·4	12·0 ± 1·1†	13·6 ± 1·5‡	12·9 ± 0·7‡	5·6 ± 0·5*	3·8 ± 0·4*	2·7 ± 0·3*	1·9 ± 0·4*
J_{30}-BSA	0·8 ± 0·2*	7·5 ± 0·8‡	8·6 ± 0·5	5·9 ± 0·7‡	8·7 ± 1·5‡	3·4 ± 1·0*	2·9 ± 0·3*	2·8 ± 0·6*	n.t.
A_{22}-BSA	1·3 ± 0·1*	4·8 ± 0·7*	4·2 ± 0·6*	12·1 ± 0·6	4·3 ± 1·0*	4·8 ± 1·0*	3·8 ± 0·7*	3·1 ± 0·6*	2·4 ± 0·5*
N_{43}-BSA	1·2 ± 0·3*	5·8 ± 1·2*	5·7 ± 0·3*	7·6 ± 0·6*	13·2 ± 1·0	5·2 ± 0·9*	n.t.	n.t.	1·5 ± 0·2*

n.t. = not tested. Sensitization and elicitation procedures were the same as those outlined in Table 4. Number of mice per group 10–25. The following symbols denote *P* values obtained by comparing the effectiveness of the homologous antigen versus that of other antigens in eliciting DH among groups of mice, all of which received the same sensitizing antigen. * $P < 0.005$; † $P < 0.025$; ‡ *P* value not significant.

Table 6. Cross reactions in delayed-type hypersensitivity between small and large haptens on an identical carrier

	Footpad swelling in 0·1 mm ± SEM Elicitation			
Sensitization	K_{23}-BSA	A_{22}-BSA	N_{43}-BSA	θ_{41}-BSA
DNP_{31}-BSA	6·1 ± 1·2‡	5·2 ± 1·7	5·3 ± 1·5	4·7 ± 0·9
ARS_7-BSA	3·4 ± 0·9	3·5 ± 0·8§	3·9 ± 0·8	2·9 ± 1·1
$SULF_{16}$-BSA	3·4 ± 0·9	2·4 ± 0·6	2·8 ± 0·8	n.t.

	Elicitation		
Sensitization	DNP_{31}-BSA	ARS_7-BSA	$SULPH_{16}$-BSA
K_{23}-BSA	12·5 ± 0·9‡	11·8 ± 1·2	13·9 ± 1·3
J_{30}-BSA	8·7 ± 0·9	8·0 ± 1·2	9·0 ± 1·8
A_{22}-BSA	4·0 ± 0·8*	7·7 ± 1·0*§	5·7 ± 1·4
N_{43}-BSA	4·2 ± 0·9†	7·9 ± 0·7†	6·1 ± 0·8

n.t. = not tested. Sensitization and elicitation procedures were the same as those outlined in Table 4. Number of mice per group: 10–20. Elicitation of groups receiving the same sensitizing antigen and sharing a common symbol * or † differ significantly from each other. $P < 0·005$, all others were not significant. In comparisons between corresponding experiments, the groups marked ‡ and § differ significantly: P value < 0·005.

DH responses were elicited 5 days later with enlarged haptens on BSA. Mice primed with DNP_{31}-BSA showed a weak response to all complexes tested (Table 6). ARS_7-BSA and $SULPH_{16}$-BSA sensitized mice for only marginal or insignificant responses. Thus, conjugates containing large haptens were never able to elicit strong DH responses in mice sensitized with the corresponding small haptens (compare Tables 4 and 6).

Sensitization with enlarged haptens resulted in strong cross reactions to the small haptens DNP, ARS and SULPH if the mice were primed with K_{23}-BSA or J_{30}-BSA, and weaker cross reactions if they were primed with A_{22}-BSA or N_{43}-BSA.

DISCUSSION

In a previous study (Snippe *et al.*, 1977) it was demonstrated that immunization with DNP–carrier complexes in DDA resulted in hapten specific DH, provided the epitope density was sufficiently high. In contrast with the response following the use of Freund's complete adjuvant (FCA) no carrier specific DH could be obtained when DDA was used as an adjuvant. Conceivably, the native carrier determinants have been effectively covered by the numerous bulky groups of bound DDA.

Extension of the use of DDA to the response to complexes with different haptens revealed DH towards all complexes with small haptens, provided the epitope density was high enough. Insertion of a spacer between hapten and carrier resulted in considerably lower (DNP or J) or slightly lower (ARS or A) DH values. No significant response was found to the non-haptenated carrier–spacer complex (G_{25}-BSA). This is in line with the results of Stashenko & Schlossman (1977) who used *in vitro* stimulation tests which showed that non-haptenated oligolysine alone was considerably less stimulatory than DNP substituted peptides. Their results demonstrated that T cells recognize a conjugate determinant composed of both the oligolysine and DNP moieties.

Treatment of mice with CY before sensitization with SRBC results in a delay in the onset but finally in an enhanced DH response (Kerckhaert, van den Berg & Willers, 1974). CY treatment preceding

immunization with hapten–carrier complexes emulsified in DDA resulted in DH responses very similar to or of only slightly lower magnitude than those obtained without CY. These results are in accordance with earlier findings in which CY pretreatment enhanced DH responses following immunization in FCA, but produced nearly full scale DH after immunization in DDA (Snippe *et al.*, 1977). These results suggest that DDA treatment favours expression of DH responses at or near optimal physiological levels.

Studies on cross reactions revealed a notable lack of specificity in many instances, for example as seen after immunization with DNP_{31}-BSA. Both small and enlarged hapten complexes elicited homologous DH responses. Sensitization with ARS_7-BSA caused cross sensitivity to $SULPH_{16}$-BSA, but sensitization with $SULPH_{16}$-BSA primed mice for significant cross reactions against ARS_7-BSA and for marginal cross reactions against DNP_{31}-BSA. Immunization with the DNP and TNP containing enlarged hapten complexes (K_{23}-BSA and J_{30}-BSA) resulted again in extensive cross reactions with enlarged and small hapten complexes. Sensitization with N_{43}-BSA, led to rather strong cross reactions with A_{22}-BSA a complex bearing a non-related hapten. Following immunization with A_{22}-BSA, only weak reactions on some of the other enlarged complexes (Table 5) and stronger reactions on ARS_7-BSA and $SULPH_{16}$-BSA were encountered (Table 6). A_{22}-BSA was the only sensitizing complex used in these experiments which had the N-acetyl-L-tyrosylglycylglycine spacer. More experiments will be necessary to draw conclusions regarding the influence of the type of spacer used on the final expression of specificity. Nonetheless, the 'immunodominant' distal terminus of the enlarged haptenic structures often failed to manifest the type of dominance in DH that is often expected for humoral immune responses.

Our results share notable similarities with the *in vitro* stimulation tests of Janeway *et al.* (1976a). They showed that lymphocytes of strain 2 guinea-pigs primed *in vivo* with conjugated mycobacteria could be stimulated *in vitro* with DNP linked to a great variety of carriers. They explained the lesser specificity of the lipid-containing conjugate used for priming on the basis of its ability to bind by non-specific hydrophobic forces to T-cell membrane lipid, thus stabilizing the weak interaction between the DNP group and any receptor even partially specific for it (Janeway & Paul, 1976b). The broad array of cross reactions in DH following immunization with complexes containing the electrostatically bound lipid groups of DDA seems to support this hypothesis.

It seems likely that the receptors which determine T-cell specificity differ substantially from those which determine B cell specificity. Moreover, any T-cell propensity for cross-reactivity or alloreactivity (Merchant & Inman, 1977) was probably further amplified in the present studies by associated lipid binding.

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