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Variable expression of delayed hypersensitivity in different mouse strains using dimethyl dioctadecyl ammonium bromide as an adjuvant

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Summary. Delayed-type hypersensitivity measured as footpad swelling was studied in a large number of inbred mouse strains. A conjugate of bovine serum albumin (BSA) with the small 2,4-dinitrophenyl (DNP) hapten served to generate strong reactions, specific for the DNP group. Delayed hypersensitivity was produced with the DNP-BSA complex mixed with the cationic, surface active lipid, dimethyl dioctadecyl ammonium bromide (DDA). Great variation was observed in delayed hypersensitivity among different mouse strains. For convenience, the mice were classified into five groups, notably: non-, low, moderate, good and high responders. The highest responding animals were BALB/cJ mice, the lowest were P/JN and outbred nu/nu mice. No correlation was observed between H-2 type and the intensity of the elicited reactions.

INTRODUCTION

Delayed hypersensitivity is classified as a cell-mediated immune phenomenon, since it can be mediated by antigen-reactive T cells. The reaction is characterized by a slowly developing induration, erythema and oedema which becomes maximal at about 24–96 h after antigen injection, depending on the species tested and the antigen used (Crowle, 1975). The hypersensitivity can be adoptively transferred in mice by lymphoid cells (Asherson & Zembala, 1973).

Previous experiments (Snippe, Belder & Willers, 1977; Snippe, Johannesen, Inman & Merchant, 1978) demonstrated that the cationic surface active lipid, dimethyl dioctadecyl ammonium bromide (DDA) is an excellent sensitizing adjuvant for generating a state of delayed hypersensitivity which can be measured by the footpad swelling test in BALB/c mice. DDA contains a positively charged quarternary ammonium group and two long hydrophobic lipid chains. When DDA is mixed with DNP-haptenated bovine serum albumin (DNP–BSA), it probably binds tightly to the negatively charged protein molecule by non-covalent bonds (Dailey & Hunter, 1974). Autoradiographic studies with ^{125}I-labelled material have demonstrated that the DDA-treated protein antigens are consistently localized in the paracortical areas of lymph nodes, in close proximity to the thymus-derived cells which are stimulated during the induction of hypersensitivity (Dailey & Hunter, 1974). The advantages of DDA over a number of other adjuvants are: (1) the absence of concomitant antibody formation; (2) induction of hypersensitivity, measured as footpad swelling which significantly exceeds the response of the same antigens in Freund's complete adjuvant (Snippe

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et al., 1977); (3) probable absence of native immunogenic components in DDA; (4) the absence of lesion formation at the injection site. In contrast, Freund's complete adjuvant commonly induces also antibody formation, it contains immunogenic components of mycobacteria, and it often causes severe local ulceration at the injection site.

Since strain differences are well known to affect immune responses, we employed DDA as an adjuvant to evoke delayed hypersensitivity in mice in order to probe for possible variations in cell-mediated immune response in different inbred mouse strains.

MATERIALS AND METHODS

Animals and immunization

Inbred mice were obtained from the Animal Production Section, National Institutes of Health, Bethesda, MD or from Jackson Laboratories, Bar Harbor, Maine and were used at 8–12 weeks of age. Outbred N:NIH(s) congenitally athymic nude (nu/nu) mice and their heterozygous (nu/+) littermates were obtained from the specific pathogen free (SPF) unit of the Small Animal Production Section at the National Institutes of Health. For each experiment, groups containing three to five mice were used. Ten micrograms of DNP_{31}-BSA dissolved in 0·1 ml saline were mixed with a suspension of 100 μg of DDA (Eastman Kodak, Rochester, NY) in 0·1 ml saline. the 0·2 ml suspension was injected intracutaneously (i.c.) on the abdomen at two separate sites.

Antigens

The 2,4-dinitrophenyl group (DNP) was coupled to bovine serum albumin (Poviet, Amsterdam, The Netherlands) using the 2,4-dinitrobenzene sulphonic acid sodium salt (Eastman Kodak, Rochester, NY) to yield DNP_{31}-BSA. The conjugation was carried out and analysed according to the method of Eisen, Carsten & Belman (1954).

Assay for delayed hypersensitivity

Delayed hypersensitivity was determined by measuring the increase in footpad thickness (footpad swelling test) as described by Kerckhaert, van den Berg & Willers (1974) after injecting 10 μg of DNP_{31}-BSA on 0·05 ml of saline into the left footpad. The response was expressed as the increment in footpad thickness, measured 24 h after the antigen injection. A footpad swelling of 0·30 mm was regarded as significant.

Haemolytic plaque assay

Immune cell suspensions and hapten-specific PFC plating, incubation, plaque development and counting conditions were identical with those previously described (Snippe, Merchant, Johannesen & Inman, 1978). Erythrocytes optimally coated with the tripeptide-enlarged hapten DNP (N-2,4-dinitrophenyl)-β-alanylglycylglycine were prepared according to the procedure of Inman, Merchant, Claflin & Tacey (1973) and were used as indicator erythrocytes in the PFC assay. The numbers of PFC were corrected for background activity. Data have been expressed as the mean numbers of PFC per spleen $\pm$ SE.

Statistical analysis

Results are expressed as the geometric mean of n values $\pm$ the standard error (SE). Student's t test was used to analyse the statistical significance of the results. P values $>0{\cdot}05$ were considered non-significant (NS).

RESULTS

Delayed hypersensitivity (DH) in related and unrelated mouse strains

Groups of mice (three to nineteen per group) were immunized i.c. with 10 μg of DNP_{31}-BSA in 100 μg of DDA and 5 days later challenged in the right hind footpad with 10μg of DNP_{31}-BSA. DH was determined by measuring the increase in footpad thickness 24 h later. From the data in Table 1 it is clear that great variation exists in DH responsiveness among the various mouse strains. The highest response was observed with BALB/cJ mice (footpad swelling (FS): $2{\cdot}43 \pm 0{\cdot}07$ mm). No DH reactions occurred with P/JN mice (FS: $0{\cdot}02 \pm 0{\cdot}02$ mm).

For convenience, the findings were grouped into five categories ranging from non-responders to high responders (Table 2). DH responsiveness appeared in general to be independent of H-2 type except that both available $H\text{-}2^p$ strains were non-responders and all three $H\text{-}2^a$ strains studied were good responders. For each of several standard mouse strains (e.g. DBA/2, AKR, C3H/He and BALB/c) substrains derived from different production colonies produced comparable levels of DH responsiveness.

Delayed hypersensitivity in F_1 hybrids

A number of different F_1 hybrids and their parent

Table 1. Delayed hypersensitivity in different mouse strains measured as footpad swelling

Strain*	H-2	Number tested	Geom. mean† ±SE	Strain	H-2	Number tested	Geom. mean ±SE
A/J	a	19	1·67±0·10	C3H/HeJ	k	18	0·72±0·06
A/HeJ	a	6	1·74±0·27	C3H/HeN	k	6	1·15±0·05
A/WySnJ	a	5	1·17±0·36	C3HF/HeN	k	5	1·15±0·04
AKR/J	k	8	1·18±0·10	DBA/1JN	q	3	0·34±0·10
AKR/N	k	5	0·76±0·24	DBA/2J	d	4	1·76±0·11
AU/SsJ	q	9	0·89±0·10	DBA/2N	d	8	1·00±0·07
BALB/cAnN	d	5	2·17±0·11	GR/N	?	7	0·60±0·11
BALB/cJ	d	5	2·43±0·07	I/StN	j	5	0·69±0·13
BDL/N	?‡	5	0·13±0·06	NBL/N	d	5	1·43±0·07
BDP/J	p	9	0·21±0·04	NGP/N	?	5	0·90±0·17
BRSUNT/N	?	5	0·70±0·08	NH/LwN	?	7	0·24±0·10
BUB/BnJ	q	4	0·21±0·19	NZB/N	d	6	0·86±0·09
B10.D2/oSnJ	d	7	1·04±0·24	NZW/N	z	6	0·40±0·14
B10.D2/nSnJ	d	4	0·97±0·23	P/JN	p	4	0·02±0·02
CBA/CaJ	k	15	0·85±0·16	RF/J	k	6	0·85±0·19
CBA/CaHN	k	7	0·51±0·11	RIII/AaN	r	6	0·65±0·09
CBA/J	k	12	0·79±0·07	SPM/N	?	5	1·31±0·16
CBA/N	k	7	0·45±0·17	ST/N	k	5	1·32±0·26
C57Bl/6J	b	27	0·30±0·06	STAR/N	?	11	0·99±0·12
C57Bl/KaLwN	?	6	0·45±0·14	STR/N	?	12	0·75±0·09
C57Bl/10ScN	b	5	0·13±0·02	SWR/J	q	8	0·24±0·20
C57Bl/10SnJ	b	6	0·68±0·16	SJl/N	s	5	0·50+0·07
C57L/J	b	10	0·29±0·09	129/SvJ	b	6	0·42±0·04
C57L/N	b	5	0·73±0·16	nu/+	?	10	0·55±0·10
C58/LwN	k	4	0·99±0·14	nu/nu	?	10	0·07±0·01

* Origins are: J. Jackson Laboratories, Bar Harbor, ME & N, National Institutes of Health, Bethesda, MD, USA.

† Footpad swelling in 1 mm units ±SE. Groups of female mice (three to twenty-seven per group) were immunized i.c. with 10 μg of DNP_{31}–BSA in 100 μg DDA and 5 days later their DH responses were elicited with 10 μg of the homologous antigen injected into the right, hind footpad.

?‡, H-2 type uncertain.

strains were tested for their DH reactivity. Both male and female mice were immunized i.c. with 10 μg of DNP_{31}-BSA in 100 μg of DDA and 5 days later footpad-challenged with 10 μg of DNP_{31}-BSA. The results are presented in Table 3. The F_1 hybrids $AKD2F_1/J$ and CAF_1/J derived from two high responder parent strains were also high responders. Crosses of a low and a high responding mouse strain resulted in an F_1 hybrid ($B6AF_1/J$) which showed moderate to high DH responsiveness. Crosses of two moderate responding strains resulted in an F_1 hybrid ($CBC3F_1/N$) which made moderate DH reactions. However, F_1 hybrids obtained from non-responder C57Bl/6J females and high responder DBA/2J males resulted in non-responder $B6D2F_1/J$, male and female progeny.

Antibody production after intracutaneous injection of antigen mixed with DDA

In previous experiments (Snippe *et al.*, 1977) a hapten-specific DH in BALB/c mice was induced without concomitant antibody formation, using DDA as adjuvant. To extend the measurements of the effects of DDA on antibody formation to other mouse strains, a number of representative mouse strains (taken from Table 2) were tested for their ability to induce antibody production after i.c. immunization of antigen in DDA. Two groups, each of five female C57Bl/6J, C3H/HeJ, AKR/J, DBA/2J and BALB/cJ mice were immunized i.c. either with 25 μg DNP_{31}-BSA or saline (controls) mixed with 100 μg of DDA. At day 5 the numbers of

Table 2. Categories of delayed hypersensitivity responsiveness in different mouse strains*

Group I		Group II		Group III		Group IV		Group V	
Non responders		Low responders		Moderate responders		Good responders		High responders	
FS† < 0·30		FS < 0·60 > 0·30		FS < 1·00 > 0·60		FS < 2·00 > 1·00		FS > 2·00	
	H-2		H-2		H-2		H-2	FS > 2·00	H-2
P/JN	p	DBA/1JN	q	nu/+	?	DBA/2N	d	BALB/cAnN	d
nu/nu	?‡	NZW/N	z	GR/N	?	C3HR/HeN	k	BALB/cJ	d
BDL/N	?	129/SvJ	b	RIII/AaN	r	C3H/HeN	k		
C57Bl/10SnJ	b	CBA/N	k	C57Bl/10SnJ	b	A/WySn	a		
BDP/J	p	C57Bl/KaLwN	?	I/StN	j	AKR/J	a		
BUB/Bnj	q	SJL/N	s	BRSUNT/N	?	SPM/N	?		
NH/LwN	?	CBA/CaHN	k	C3H/HeJ	k	ST/N	k		
SWR/J	q			C57L/N	b	NBL/N	d		
C57L/j	b			STR/N	?	A/J	a		
C57Bl/6J	b			AKR/N	k	A/HeJ	a		
				CBA/J	k	DBA/2J	d		
				RF/J	k				
				CBA/CaJ	k				
				NZB/N	d				
				AU/SsJ	q				
				NGP/N	?				
				B10.D2/nSnJ	d				
				STAR/N	?				
				C58/LwN	k				
				B10.D2/oSnJ	d				

* Put in order of lowest to highest responsiveness.
† FS, footpad swelling in 1 mm units. For legends see Table 1 and text.
?‡, H-2 type uncertain.

Table 3. Delayed hypersensitivity in parental mouse strains and their F_1 hybrids

Strains	Footpad swelling in 1 mm ±SE	
	Male	Female
Parents		
A/J	0·87±0·07	1·17±0·10
AKR/J	0·63±0·19	1·18±0·10
BALB/cJ	NT*	2·43±0·07
CBA/N	0·58±0·28	0·45±0·17
C57Bl/6J	0·31±0·07	0·27±0·04
C3H/HeN	1·01±0·06	1·15±0·05
DBA/2J	1·07±0·08	1·76±0·11
F_1 hybrids		
AKD2F_1/J (AKR/J ♀× DBA/2J ♂)	1·35±0·17	1·46±0·15
CAF_1/J (BALB/cJ ♀× A/J ♂)	1·17±0·03	1·19±0·09
B6AF_1/J (C57Bl/6J ♀× A/J ♂)	0·77±0·09	0·93±0·23
CBC3F_1/N (CBA/N ♀× C_3H/HeN♂)	0·55±0·13	0·62±0·14
B6D2F_1/J (C57Bl/6J ♀× DBA/2J ♂)	0·31±0·09	0·25±0·06

* NT, not tested.

Groups of mice (five to seventeen per group) were immunized i.c. with 10 μg of DNP_{31}-BSA in 100 μg of DDA and 5 days later their footpad responses were elicited with 10μg of DNP_{31}–BSA.

direct DNP-reactive PFC were determined in the mouse spleens. In no instance were significant differences between immunized and control mice found (Table 4).

Table 4. DNP-reactive plaque-forming cells in the spleens of different mouse strains after intracutaneous injection of DNP_{31}–BSA in DDA

	DH response*	Direct DNP-reactive PFC/spleen ±SE	
		control	immunized
C57Bl/6J	Non	82±26	94±18
C3H/HeJ	Moderate	460±76	380±39
AKR/J	Good	180±31	225±53
DBA/2J	Good	1940±146	1848±183
BALB/cJ	High	1332±180	1264±228

Groups of five mice were immunized i.c. with 25 μg DNP_{31}–BSA or saline (controls) in 100 μg of DDA. At day 5 the numbers of direct DNP-reactive PFC were determined in the spleens.

* Taken from Table 2.

DISCUSSION

Delayed-type hypersensitivity, it is accepted, can be measured by footpad swelling *in vivo* (Askenase, Hayden & Gershon, 1977; Mitsuoka, Teramatsu, Baba, Morikawa & Yasuhira, 1978, reviewed by Crowle, 1975). The very limited capacity of mice to produce delayed hypersensitivity *in vivo* (Brent, Brown & Medawar, 1962) can be enhanced appreciably by modifying the dose and route of antigen injection (Kerckhaert, 1974; Lagrange, Mackaness & Miller, 1974) or by use of Freund's complete adjuvant (Crowle, 1975).

Coon & Hunter (1973, 1975) used BSA heavily conjugated with fatty acids to induce in guinea-pigs strong, sustained delayed hypersensitivity to BSA without detectable antibody formation. The fatty acids needed to be covalently linked to BSA; the mere mixing of the two did not detectably change the immunogenic properties of BSA. Double conjugation of BSA with fatty acids and DNP produced delayed hypersensitivity exclusively to the hapten (Dailey & Hunter, 1974). These authors obtained similar results by mixing the antigen with the cationic surface-active

lipid DDA, and we (Snippe *et al.*, 1977) showed that DDA acts as an excellent adjuvant in BALB/c mice predisposing to the development of strong delayed hypersensitivity to haptenic groups without detectable antibody formation.

Although it is well known that strain differences and ageing affect the immune response, few studies have compared delayed hypersensitivity in different mouse strains (Mitsuoka *et al.*, 1978). We tested DDA as an adjuvant in fifty different mouse strains and found great variations in the magnitude of delayed hypersensitivity responses among the different strains. The highest responders were the BALB/cJ mice, and the lowest were P/JN mice. Preliminary breeding experiments (Table 3) with inbred mouse strains did not permit a full analysis of the genetic control of DH to the DNP–BSA–DDA complex being studied. However, the non-responder offspring from the matings of high-responder and low-responder strains indicates that the ability to respond to DNP–BSA–DDA is not inherited as a single autosomal dominant gene, but that additional genetic factors are involved.

We have classified general murine DH responsiveness into five arbitrarily divided groups: non-, low, moderate, good and high responders (Table 2). Several mouse strains were tested by Mitsuoka and his colleagues (1978) for the extent of their DH responsiveness to SRBC. They found C57Bl > AKR > BALB/c > C3H. This order of responsiveness is nearly opposite to the order that we observed. The difference may be attributable to differences in the eliciting antigens or in the augmenting adjuvant. If significant antibody responses are being produced against SRBC concomitant to DH production, such antibodies could also substantially interfere with DH expression. In our system no antibody production as measured with the haemolytic plaque procedure could be detected (Table 4).

Our results do not support the concept that histocompatibility-linked immune response (Ir) genes are important for the overall expression of delayed hypersensitivity in mice, although such is observed in guinea-pigs (McDevitt & Benacerraf, 1969). A possible explanation for this failure to demonstrate a relationship between H-2 and Ir genes for DH in mice might relate to the complexity of the BSA carrier used in our study. The findings of previous workers point to a major role for the carrier in detecting an H-2 restriction in DH-related responses (Green, Paul & Benacerraf, 1968).

Genes affecting T-cell functions can profoundly influence delayed-type hypersensitivity. This is currently seen with athymic nude mice. Evidence of faulty macrophage function exists in P/JN mice (Borashi & Meltzer, 1979). Such a defect might be responsible for their virtual lack of delayed hypersensitivity in our present studies.

Biozzi, Stiffel, Mouton, Bouthillier & Decreusfond (1972) who immunized outbred mice with SRBC and selected for high and low responders found that antibody responses are under the control of eight to ten different loci whose cumulative effects give rise to the characteristic being selected for. Wiener & Bandieri (1974) claimed that one or more loci control antigen handling by macrophages. They found that antigen is more readily rendered non-immunogenic in the low-responder line, than in the high-responder line. Excessive antigen catabolism by macrophages could also account for some of our results. Refractoriness of macrophages to lymphokine-mediated stimuli might also yield reduced delayed hypersensitivity. The role of macrophages in our system (DDA-induced DH) is also unknown both in the induction phase and in the elicitation phase. New approaches to study murine DH are necessary to clarify the role of macrophages in this reaction.

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