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QUANTITATIVE DINITROCHLOROBENZENE CONTACT SENSITIZATION IN A NORMAL POPULATION

W. J. CATALONA, P. T. TAYLOR AND P. B. CHRETIEN

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Surgery Branch, National Cancer Institute, National Cancer Institute, N.I.H., Bethesda, U.S.A.

SUMMARY

Using a quantitative method based on the sponteneous flare phenomenon, contact sensitization to DNCB was studied in 143 healthy volunteers between the ages of 20 and 80 yr. A spontaneous flare reaction occurred in 96·5% of subjects tested, regardless of age, and a correlation was demonstrated between the intensity of the primary response to DNCB and the threshold dose of DNCB required to elicit an anamnestic response. The results are in striking contrast to a 40% incidence of spontaneous flare reactions previously found in cancer patients using the same method. These findings show that this method of assaying reactivity to DNCB detects abnormalities of cell-mediated immunity not demonstrated by qualitative methods.

INTRODUCTION

Altered immunologic responsiveness has been demonstrated in a variety of disease states in man, including infectious diseases (Mitchell *et al.*, 1928; Gross, 1964; Starr & Berkovitch, 1964; Turk & Waters, 1969), sarcoidosis (Jones, 1967; Sharma, James & Fox, 1971), Crohn's disease (Walker & Greaves, 1969), primary biliary cirrhosis (Fox *et al.*, 1969) and both lymphoid malignancies (Rostenberg, McCraney & Bluefarb, 1956; Epstein, 1958; Sokal & Primikirios, 1961; Lamb *et al.*, 1962; Aisenberg, 1962; Cone & Uhr, 1964; Levin *et al.*, 1964; Hersh & Oppenheim, 1965; Brown *et al.*, 1967) and non-lymphoid malignancies (Levin *et al.*, 1964; Solowey & Rapaport, 1965; Gross, 1965; Hughes & McKay, 1965; Krant *et al.*, 1968; Eilber & Morton, 1970). Accumulating evidence also suggests that the course of malignant diseases is influenced by host immune responsiveness (Krant *et al.*, 1968; Eilber & Morton, 1970; Cheema & Hersh, 1971; Hersh *et al.*, 1971). Hence, it is increasingly important that improved methods of quantitating immune reactivity be developed.

While sensitive techniques have been developed for the quantitation of humoral antibody

Correspondence: Dr W. J. Catalona, Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014, U.S.A.

responses, methods of measuring cell-mediated immunity are crude by comparison. The elicitation of delayed cutaneous hypersensitivity (DCH) reactions to intradermally injected antigens has been employed widely to measure cell-mediated immunity *in vivo*. However, these antigens yield limited information concerning cellular immune responsiveness, since most are complex biologic extracts which incite a combination of both humoral and cellular immune responses. Furthermore, they are usually employed to elicit anamnestic responses which rely upon previous exposure to the allergen. Thus, they provide information regarding only the central and efferent limbs of the immune response and not the ability of an individual to respond to a newly encountered antigen.

The limitations of intradermal DCH tests are obviated by the use of a contact sensitizing agent such as 2,4-dinitrochlorobenzene (DNCB). The agent is an allergen of definite, known chemical constitution; therefore, both sensitization and challenge can be accurately controlled in terms of time and dose, and experimental conditions are exactly reproducible. Humoral antibodies are not involved in DCH reactions to *topical* DNCB (Waksman, 1960). Thus, it is a specific test for cell-mediated immunity that assesses the current ability of a subject to manifest DCH to a newly encountered antigen.

Recently, we have described a method for quantitating DCH to DNCB (Catalona *et al.*, 1972) that revealed a spectrum of reactivities in cancer patients which had not been shown with conventional methods of DNCB skin testing. The present report is concerned with the assay of DNCB reactivity in a large population of healthy subjects using this method. This study, and comparisons with the study of cancer patients (Catalona *et al.*, 1972), demonstrated that this test system quantitates DCH to DNCB and is a significant refinement over previously reported methods.

MATERIALS AND METHODS

Volunteers were solicited from employees of the National Institutes of Health, Bethesda, Maryland, and from residents of a nearby retirement community. Subjects with a history of cancer or who were taking any medication known to alter inflammatory or immune responses were excluded from the study. All were ambulatory and in good general health. A total of 143 people were studied. Fifty-four per cent were females and 46% were males; 12% were Negroes. Their ages ranged from 20 to 80 yr, being nearly equally distributed in each decade represented (Table 1).

Sealed 1-ml ampules containing 2000 μg and 50 μg solutions of DNCB per 0·1 ml in acetone were prepared by the Pharmaceutical Development Service of the National Institutes of Health. The ampules were opened immediately before use and the remainder discarded afterwards to avoid variations in concentration due to evaporation of acetone. The ampules were stored at $-20°C$ in the dark until used. Serial dilutions ranging from 1·3 to 75 μg/0·1 ml were also prepared for studies of the threshold concentration of DNCB necessary to produce an anamnestic response.

The volar aspect of the arm and forearm was cleansed with acetone. A stainless steel ring 2 cm in diameter with an affixed handle was placed so that the test site was horizontal and 0·1 ml of the test solution was applied with a volumetric dispenser.* 2000 μg were applied to the site on the upper arm and 50 μg to the volar forearm. Evaporation of the solvent

* Biopette, Schwartz-Mann, Catalog No. 0010-19 and 0010-20.

was hastened under an air stream from a portable hair dryer. Both sites were covered with a commercial bandage strip for 24 hr.

The method of scoring DCH to DNCB was the same used in the previous study (Catalona *et al.*, 1972). All subjects were examined daily for a minimum of 16 days and the reactions scored on the 14th and 16th days. The quantitative method employed for scoring the reaction incorporates spontaneous flare reactions (circumscribed erythema and a distinct margin of induration) to the residual of the sensitizing doses remaining in the skin. A spontaneous flare reaction at both the 2000-μg and 50-μg sites by the 14th day after the sensitization is graded 4+. A spontaneous flare at the 2000 μg site alone and either no reaction or an equivocal reaction at the 50 μg site is graded 3+. In the absence of a spontaneous flare at the 2000-μg site, or if the reaction at this dose is equivocal by 14 days, a challenge dose of 50 μg is applied to the volar forearm. This site is examined 24 and 48 hr later for evidence of DCH. An unequivocal DCH reaction upon challenge is graded 2+. Any equivocal reaction upon challenge is biopsied and examined for the histologic features of DCH. If these are present, the reaction is graded 1+.

Approximately 2 months following sensitization, twenty subjects were selected for study of the threshold dose of DNCB necessary to elicit an anamnestic response. Subjects with 4+ primary responses were challenged with 1·3, 2·5, and 5·0 μg doses. Subjects with spontaneous flares at the 50 μg dose occurring later than 14 days after sensitization, termed late 4+ primary response, and those with 3+ primary responses were challenged with 5, 12·5, 25, 50, and 75 μg of DNCB. Correlations were made between the reactivity observed with the primary response and the dose of DNCB required to elicit an anamnestic response.

The incidence and distribution of reactivities to DNCB were compared with age, sex, race, family history of cancer, history of allergy and the concurrent use of medications, alcohol and tobacco. Chi-square analysis was employed to determine the significance of the differences observed relative to these variables. The standard error of the difference of the percentages (SEDP) was employed to determine the significance of the relationship between the development of a spontaneous flare at the 50-μg test site (4+ reactivity) and these variables. Correlation coefficients were used to measure the degree of association between the characteristics of the population.

RESULTS

Of the 143 subjects tested, 139 (97·2%) were sensitized to DNCB by the criteria used. All but one of the sensitized subjects exhibited either a 3+ or 4+ reaction (Table 1). The mean interval between sensitization and a spontaneous flare reaction was 9–10 days for the 2000-μg dose and 11–12 days for the 50-μg dose (Table 2). This did not vary significantly with age.

Age

Although 96·5% of the subjects developed a spontaneous flare at the 2000-μg dose site by 14 days after sensitization, only 39·2% also developed a flare at the 50-μg dose site within this time. However, an additional thirty-nine subjects (27%) developed a spontaneous flare reaction at the 50-μg site 15–28 days after sensitization. Subjects in this group were termed 'late 4+ reactors'. The incidence of 3+ and 4+ reactors by 14 days did not differ with age, but the incidence of late 4+ reactors was high in the young and low in the aged. Therefore,

when the 4+ and 'late 4+' reactors are combined to form an 'ultimate 4+' group, there is an association between a decline in spontaneous reactivity at the 50-μg site and increasing age which is significant at a 95% level of confidence (CC = −0·93; SE = 0·45) (Table 1).

Four subjects were scored anergic to DNCB. They were all over 40 yr of age, but were equally distributed throughout the last four age decades studied. Two of the four

TABLE 1. Quantitative DNCB reactivity in 143 subjects from 20–80 yr in age

Age group	No.	Anergic (%)	1+(%)	2+(%)	3+(%)	4+(%)	Total† 3 and 4+	Ultimate 4+*
20–30	22	0	0	4	55	41	96	86
30–40	21	0	0	0	38	62	100	86
40–50	25	4	0	0	48	48	96	72
50–60	26	4	0	0	73	23	96	58
60–70	28	4	0	0	57	39	96	64
70–80	21	5	0	0	71	24	95	38
Mean		2·8	0·0	0·7	57·3	39·2	96·5	66·4

* Incidence correlates inversely with age (CC = −0·93, SE = 0·45, $P < 0·05$)
† Response scored 14 days after sensitization.

TABLE 2. DNCB reactivity: correlation between age and interval (in days) from sensitization to spontaneous flare

Age	Interval from sensitization to flare at 2000-μg site (days)		Interval from sensitization to flare at 50-μg site (days)	
	Mean	Median	Mean	Median
20–30	9·3	9·6	11·0	11·5
30–40	9·2	9·8	11·8	12·5
40–50	8·9	10·1	11·3	11·8
50–60	9·1	10·4	11·3	10·7
60–70	9·3	9·9	12·9	13·6
70–80	9·6	10·0	11·0	11·5

developed a DCH reaction 90 hr after challenge. This association between anergy to DNCB and increasing age approaches, but does not attain, statistical significance (CC = +0·87; SE = 0·45).

Recall threshold studies

Twenty subjects were tested with varying concentrations of DNCB to determine the threshold dose required for an anamnestic response (Table 3). All eight subjects studied who had primary 4+ reactions developed anamnestic DCH reactions upon challenge with 5 μg of DNCB. Five of these responded to 2·5 μg and three responded to 1·3 μg. Only two of six subjects with 'late 4+' primary reactions developed DCH upon challenge with 5 μg;

the remainder had thresholds varying from 12·5 to 75 μg. Similarly, only one of six subjects with a primary 3+ reaction was able to respond to 12·5 μg of DNCB. The mean threshold response of 4+ reactors was significantly lower than that of the 3+ reactors ($P<0·01$), with the threshold of the 'late 4+' reactors occurring at an intermediate position (Table 3).

TABLE 3. Correlation between the primary response to DNCB and the minimal dose of DNCB that elicited an anamnestic response

Number of subjects	Primary response to DNCB	DNCB required for anamnestic response (mean ± S.E.) (μg)
8	4+	3·2 ± 0·65*°
6	late 4+	16·3 ± 6·91°+
6	3+	45·8 ± 10·5*+

* Values differ significantly ($P<0·01$, t-test).
°+ Values differ significantly ($P<0·05$, t-test).

TABLE 4. Quantitative DNCB reactivity: correlations

Group	No.	Total subjects (%)	Anergic (%)	1+(%)	2+(%)	3+(%)	4+(%)	Total† 3+ and 4+	Ultimate (%) 4+
Allergic history	44	30·8	5	0	3	45	47	92	77
Family history of cancer	75	52·4	3	0	1	55	41	96	64
On medication	54	37·8	0	0	2	65	33	98	59
Use of alcohol	85	59·4	2	0	1	60	37	97	68
Use of tobacco	46	32·2	4	0	2	50	44	94	72
Irritative reaction to 50 μg	11	7·7	0	0	0	36	64	100	91*
Females	78	54·5	3	0	1	59	37	96	71
Males	65	45·5	3	0	0	55	42	97	62
Negroes	17	11·8	0	0	0	59	41	100	82
Caucasians	126	88·2	3	0	1	57	39	96	65
Mean			2·8	0·0	0·7	57·3	39·2	96·0	66·4

* Differs from the group as a whole ($P<0·01$, standard error of the difference of the percentages).
† Response scored at 14 days post-sensitization.

Irritative reaction

Eleven subjects developed an irritative reaction at the 50-μg dose site. Ten of the eleven (91%) also developed a spontaneous flare reaction at this site (4+ and 'late 4+'). By contrast, only 64·4% of individuals who failed to develop an irritative reaction to the 50-μg dose subsequently developed a spontaneous flare at this site (Table 4). These differences are significant ($P<0·01$).

Effect of medications

Over one-half of the subjects in the last three decades studied were taking medication during the study, compared with only 16% of the subjects from the first three decades. Although a trend toward lower reactivity was observed in patients on medication (59% ultimate 4+ versus 71% in those not on medications), the differences in the distribution of reactivities to DNCB in these two groups were not statistically significant when compared to each other or the population as a whole.

Race, allergy, family history of cancer, alcohol, tobacco

Although a trend toward higher reactivity was observed in subjects with a history of allergy, no statistically significant difference could be established. Similarly, no correlation could be demonstrated between reactivity to DNCB and race, family history of cancer, the use of alcohol or the use of tobacco (Table 4).

DISCUSSION

DNCB was first used to induce contact sensitization in humans in 1935 (Wedroff & Dolgoff, 1935). Since that time, a number of studies of the incidence of DNCB reactivity in 'control' populations have been reported (Sulzberger & Baer, 1938; Rostenberg & Kanof, 1941; Epstein, 1958; Kligman & Epstein, 1959; Waldorf *et al.*, 1968). Many different techniques were employed in these studies. The concentrations of DNCB used to induce sensitization ranged from 10 μg (Rostenberg & Kanof, 1941) to 25,000 μg (Kligman & Epstein, 1959). Similarly, doses used to elicit an anamnestic response ranged from 0·25 μg (Epstein, 1958) to 1500 μg (Sulzberger & Baer, 1938). The incidence of sensitization to DNCB reported in healthy controls varies from 58% (Rostenberg & Kanof, 1941) to 100% (Aisenberg, 1962), with most studies finding an incidence of sensitization of approximately 90% (Kligman & Epstein, 1959; Gross, 1965; Brown *et al.*, 1967; Waldorf *et al.*, 1968). Attempts to quantitate reactivity were directed at grading the intensity of the reaction which occurred upon challenge 2–4 weeks after sensitization. Because this method of scoring was found to be subjective in all studies, the results ultimately were reduced to simply 'percent reactive' and 'percent anergic'.

The fundamental nature of the spontaneous flare reaction, which develops 7–28 days later at the site of a sensitizing dose of DNCB was appreciated in 1938 (Sulzberger & Baer, 1938). The method used in the present study utilizes the spontaneous flare reaction to quantitate the levels of reactivity to DNCB. Simultaneous application of both strong (2000 μg) and weak (50 μg) doses of DNCB at different sites allows the distinction between individuals capable of exhibiting a spontaneous flare reaction within 14 days to the residual of both doses (4+), these capable of exhibiting a spontaneous flare only to the residual of the stronger dose (3+) and those incapable of a flare at either dose. That this scoring system distinguishes between quantitative differences in reactivity to DNCB is supported by the study of the threshold dose of DNCB required to elicit an anamnestic response. Individuals responding with a flare at the 50-μg site within 14 days (4+) had lower thresholds than those who required more than 14 days to flare at this site ('late 4+') or those able to display spontaneous reactivity only at the 2000-μg dose site (3+).

Every subject tested, except one, developed a pronounced irritative reaction at the 2000-μg

dose site within 24 hr of DNCB application. The subject who failed to develop an irritative reaction at this site also failed to exhibit DCH to DNCB. Previous studies have demonstrated that irritation enhances the development of sensitization (Kligman & Epstein, 1959). These studies show that in individuals who are anergic and also fail to develop an inflammatory response, it cannot be determined whether the absence of DCH response is due to a specific impairment of cell-mediated immunity or whether it is due merely to a poverty of the general inflammatory responses. Other findings in this study reflect the relationship of these two responses. Of the eleven individuals who developed an irritative reaction to the 50-μg dose, more than 90% subsequently developed a spontaneous flare at this dose, compared with only 64·4% of those who did not develop an irritative reaction at this site. Two interpretations of this association are immediately evident. Either a heightened inflammatory response evokes a more intense immune response, or the functional levels of the two systems parallel each other.

Opinions regarding the effect of ageing on immunologic phenomena in humans are conflicting. Sabin *et al.* (1947) demonstrated that antibody responses to encephalitis vaccine declined with age (Sabin *et al.*, 1947), but Brenner *et al.* (1951) found no difference in antibody response to typhoid vaccine in individuals 15–78 yr of age (Brenner *et al.*, 1951). Gross reported diminished reactivity to DNCB in elderly patients; however, all patients in his study over the age of 40 had either a chronic disease, active tuberculosis or a neoplasm (Gross, 1965). Waldorf *et al.* (1968) found that the incidence of responsiveness to DNCB declined from 95% in subjects under the age of 70 yr to 68% in healthy subjects over the age of 70 (Waldorf *et al.*, 1968). The doses of DNCB used in their study were similar to those used in the present one. In contrast to our use of the spontaneous flare reaction, however, they used as criteria for reactivity erythema, oedema and vesiculation at the site of challenge doses of 100 and 50 μg applied 14 days after the sensitizing dose of 2000 μg.

In the present study, more than 96% of all subjects tested, including individuals between the ages of 70 and 80 years of age, manifested a spontaneous flare at the 2000 μg dose (3+ or greater). The incidence of spontaneous reactivity at the 50-μg test site (4+ and 'late 4+'), however, declined with age. Thus, while one can induce contact sensitization to DNCB almost uniformly in normal subjects of all ages, responsiveness to this agent manifested by a spontaneous flare at the 50-μg dose site declines significantly after the fifth decade. However, this decline could be due, at least in part, to alterations in the blood supply or other properties of the skin associated with ageing rather than a decline in immunologic reactivity, since the day of onset of spontaneous flares in the oldest subjects was not different from the younger ones (Table 2). Furthermore, there was no significant concentration of anergic subjects in the oldest age group studied. Four persons in the study were scored anergic, and they were all over 40 years of age, but two of the four subsequently developed late reactions at the challenge site 90 hr after challenge.

Individuals taking anti-inflammatory or immunosuppressive medications were excluded from this study; however, more than one-third were taking medications of some type, particularly the older age groups. Many of these were taking antihypertensive agents, diuretics and digitalis preparations. It cannot be excluded that the trend toward lower reactivity in subjects on medications (Table 4) is due to the high relative representation of the elderly in this group, or the decline in reactivity with age ('ultimate 4+') is due in part perhaps to the use of medications.

Previous investigators have suggested that Negroes are less susceptible to contact

sensitization to DNCB than Caucasians (Epstein, 1958; Rostenberg & Kanof, 1941). In the present study, however, no significant difference between races was demonstrated. The trend toward a greater incidence of individuals responding with a flare to the 50-μg dose among Negroes (Table 4) may merely reflect the decreased representation of Negroes in the older age groups studied.

Sulzberger and Rostenberg reported an increased susceptibility to experimental sensitization to DNCB in patients with contact dermatitis (Sulzberger & Rostenberg, 1939); however, the incidence of sensitization achieved in their study was only 50%. In the present study, there was a tendency toward higher levels of reactivity in subjects with a previous history of allergy, but this difference was not statistically significant.

The results of the present study, when compared with the results of a previously reported study of cancer patients using the same technique of quantitating DNCB reactivity (Catalona *et al.*, 1972), demonstrate the advantage offered over qualitative methods for measuring DCH to DNCB. While the overwhelming majority of healthy individuals tested were capable of responding with a spontaneous flare to one or both doses of DNCB (less than 4% required challenge), of twenty patients with cancer, 60% failed to exhibit a spontaneous flare. Forty-five percent of the patients displayed DCH only after challenge (2+ and 1+) and 15% were anergic. The 45% with 2+ and 1+ reactions would be considered 'normal' by criteria of DNCB reactivity used in previous studies; however, their reactivity is clearly subnormal by the norms established with the quantitative method used in this study.

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