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STUDIES ON THE CONTACT SENSITIZATION OF MAN WITH SIMPLE CHEMICALS

III. Quantitative Relationships Between Specific Lymphocyte Transformation, Skin Sensitivity, and Lymphokine Activity in Response to Dinitrochlorobenzene

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Dinitrochlorobenzene (DNCB) coupled to peripheral blood erythrocytes or leukocytes forms a particulate complex, DNCB–antigen. The addition of DNCB–antigen induced blastogenesis and DNA synthesis in leukocyte cultures from DNCB-sensitized human subjects and not in leukocyte cultures from nonsensitized controls. In general, sensitized subjects who displayed a higher degree of cutaneous reactivity to DNCB, as manifested by duration and intensity of dermatitits, also showed a greater blastogenic response to DNCB–antigen in vitro. This quantitative correlation, however, was not invariant. Certain soluble factor(s), or lymphokines are released following the addition of DNCB–antigen to leukocyte cultures prepared from some sensitive subjects who were rechallenged one or more times with DNCB. These lymphokines induce blastogenesis in secondary target leukocyte populations from nonsensitized subjects. Extended studies are presented which show little or no lymphokine activity in peripheral blood leukocyte cultures during a primary immune response, despite high degrees of blastogenic activity in response to DNCB–antigen. Significant lymphokine activity was observed only following additional rechallenge with DNCB.

Blastogenesis and skin reactivity specific for DNCB have been shown to develop at about the same time during a primary immune response. This, along with the quantitative correlation shown in this communication, suggests that both processes probably reflect thymic-dependent cellular immunity. The appearance of lymphokine activity following rechallenge with DNCB suggests that DNCB-induced lymphokines may represent an amplifying mechanism of the cellular immune response that involves recruitment of previously uncommitted lymphocytes.

Allergic contact dermatitis to dinitrochlorobenzene is generally accepted as a manifestation of cell-mediated immunity (CMI) [1,2]. Lymphocyte transformation has also been reported as a frequent in vitro correlate or manifestation of CMI [3,5]. We have previously reported a specific blastogenic response to DNCB complexed to leukocytes [6] or erythrocytes [7]. These DNCB complexes are referred to as DNCB–antigen and induce blastogenesis and DNA synthesis only in leukocyte cultures from DNCB-sensitized subjects. Extensions of these studies indicate that lymphokine(s) is specifically induced in sensitive leukocyte cultures by DNCB–antigen [8]. These DNCB-induced lymphokines are unrelated to histocompatibility factors and induce similar degrees of blastogenesis in autologous-sensitive and allogeneic and syngeneic leukocyte cultures from nonsensitized subjects [9]. The blastogenic activity therefore appears similar to cell-free soluble factors referred to as "lymphokines" which have been implicated as possible mediators of cellular immunity [10].

In previous studies we have presented over 30 experiments in which nonsensitized subjects showed no significant response to DNCB–antigen while, simultaneously, sensitized subjects showed from 4- to over 100-fold increases in DNA synthesis [6,7]. In this report we illustrate a quantitative relationship between the intensity and duration of cutaneous inflammation to DNCB in vivo, and lymphocyte transformation to DNCB–antigen in vitro. In addition, little or no lymphokine activity was observed in 12 subjects studied following a primary sensitization with DNCB even with high levels of blastogenesis to DNCB–antigen. Only after additional rechallenge with DNCB was significant lymphokine activity observed.

Since specific blastogenesis and skin reactivity to DNCB convert at about the same time during a

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primary immune response and show a quantitative relationship, it seems probable that both are a reflection of thymic-dependent cellular immunity. Lymphokine activity, manifested by cell-free soluble blastogenic factor(s) released in DNCB-antigen-stimulated sensitive leukocyte cultures, may represent an amplifying mechanism of the cellular immune response involving recruitment of previously uncommitted lymphocytes.

MATERIALS AND METHODS

Sensitization and patch testing with DNCB. A sensitizing dose of 2,000 μg of DNCB in 0.1 ml of acetone was applied to the skin of the medial aspect of both upper arms within a 2-cm diameter polyethylene ring, allowed to evaporate, and covered by an adhesive bandage for 2 days. Simultaneously 50 μg and 5 μg of DNCB in acetone were applied to one forearm [2,7]. On days 3, 6, and 9 following application of the sensitizing dose, subjects were challenged with 200 μg, 10 μg, and 1 μg of DNCB in a cream base [Acid Mantle Creme (aluminum acetate), Dome Laboratories, Westhaven, Conn.] under partial occlusion (Johnson & Johnson Sheer patches) for 2 days.

Subjects and recording of cutaneous reactivity to DNCB. Healthy adult volunteers were sensitized in the above manner and cutaneous reactivity to DNCB was recorded daily at each of the patch-test sites. Erythema alone was recorded as 1+, erythema and induration as 2+, vesicle formation as 3+.

Preparation of DNCB-antigen. We have previously described in detail the preparation of DNCB complexes used for in vitro stimulation. These complexes can be prepared with leukocytes [6] or erythrocytes [7] and form nontoxic, storable, particulate complexes referred to as DNCB-antigen. In the current study all DNCB-antigen used was prepared with erythrocytes from a type O-positive donor. Briefly, 3 ml of packed erythrocytes was suspended in 40 ml of undiluted DMSO (dimethyl sulfoxide, Aldrich Chemical Co., Inc, Milwaukee, Wisconsin) containing 1 gm/100 ml of DNCB (1-chloro-2,4-dinitrobenzene, Eastman Kodak Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.). After 1-hr incubation at 38°C the cell pellet was washed once with DMSO, and twice with Medium 199, lyophilized, and stored in the dark at 4°C.

Preparation of leukocyte cultures and addition of DNCB-antigen. "Mononuclear leukocyte culture fluid" was prepared by diluting the "mononuclear leukocyte-rich plasma" obtained by centrifugation [11] with 4 parts of tissue culture medium (RPMI 1640 Grand Island Biological, 1876) containing penicillin and streptomycin. This method yields a final concentration of 0.4–2 × 10^6 cells/ml of which 70–90% are lymphocytes [11].

Lyophilized DNCB-antigen prepared from erythrocytes as above was suspended in "cell-free" culture fluid" (20% plasma in RPMI 1640) at a concentration of 250 μg/ml and mixed 1:9 with mononuclear leukocyte culture fluid to give a final concentration of 25 μg DNCB-antigen per ml of culture. An equal volume of cell-free culture fluid without DNCB-antigen was added to unstimulated control cultures. The plasma was the same for all stimulated and unstimulated cultures within each experiment. All cultures were cultured in 0.5-ml volumes at 38°C with room air as the gas phase in 12 × 35 mm screw-capped vials (Arthur H. Thomas Co., Philadelphia, Pa.).

Lymphokine supernate harvest and assay. Details of this procedure are published elsewhere [8]. In brief, cultures for the production of lymphokine(s) were prepared by culturing leukocytes alone (unstimulated control cultures) or in the presence of DNCB-antigen (DNCB-antigen stimulated cultures). These primary cultures were obtained during the third week following sensitization or rechallenge with DNCB. Detailed studies of the kinetics of lymphokine production have shown this to be an appropriate time to measure lymphokine activity (manuscript in preparation). These cells were cultured in 5-ml volumes at 38°C with room air as the gas phase in 2-oz prescription flasks. In studies presented in this report, supernates from these cultures were harvested by centrifugation between 46 to 51 hr and were filtered through 0.45-μ Millipore filters; 0.5-ml aliquots of the primary cultures were removed at the time of supernate harvest, placed in 12 × 35 mm screw-capped vials (Arthur H. Thomas Co.), and assayed for DNA synthesis. Assay of lymphokine activity involved the addition of supernates of stimulated and unstimulated primary cultures to secondary leukocyte cultures prepared from DNCB-insensitive subjects. The concentration of leukocytes was maintained constant within each experiment. Secondary target leukocyte cultures were cultured in 0.5-ml volumes at 38°C with room air as the gas phase in 12 × 35 mm screw-capped vials.

Evaluation of cultures. Tritiated thymidine incorporation during DNA synthesis was measured between the 115th and 125th hr. In each experiment, duplicate or triplicate cultures were assayed at the same time and the

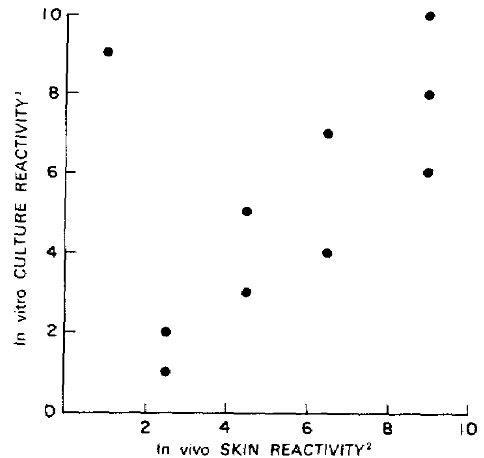

FIGURE. Correlation between DNCB skin sensitivity in vivo and specific lymphocyte transformation in vitro.

[1] In vitro lymphocyte transformation in leukocyte cultures prepared from 10 subjects 20 days following application of a primary sensitizing dose of DNCB. Plot represents rank order from 1 to 10 of stimulation ratios

$$\frac{\text{DNCB-antigen-stimulated (cpm)}}{\text{unstimulated control (cpm)}}$$

[2] In vivo skin test reactivity of these 10 subjects on day 20 following application of a primary sensitizing dose of DNCB. Plot represents rank order of intensity of dilutional patch test applied on day 6.

TABLE I. *Detailed tabulation in vitro and in vivo data on 10 DNCB-sensitized subjects*

Subjects	In vitro culture reactivity[a]				In vivo skin reactivity[b]		
	DNA synthesis in CPM		Ratio				
	Unstimulated control	DNCB-Ag-stimulated	Stimulated/control	Rank	200 μg	10 μg	Rank
T.J.	362 ± 166	11233 ± 385	31.0	10	2+	2+	9
A.L.	281 ± 11	8384	28.9	9	0	0	1
S.S.	3445 ± 1073	77089 ± 1719	22.4	8	2+	2+	9
W.B.	897 ± 421	7624 ± 638	8.5	7	2+	1+	6.5
D.T.	994 ± 418	6591 ± 132	6.6	6	2+	2+	9
T.A.	480 ± 26	2727 ± 279	5.7	5	2+	0	4.5
R.F.	489 ± 7	2308 ± 154	4.7	4	2+	1+	6.5
J.D.	4910	10251 ± 763	2.1	3	2+	0	4.5
R.C.	643 ± 95	907 ± 363	1.4	2	1+	0	2.5
E.A.	199 ± 9	219 ± 9	1.1	1	1+	0	2.5

[a] Leukocyte cultures prepared on day 20 following application of a primary sensitizing dose of DNCB.

[b] Intensity of skin response 20 days following application of a primary sensitizing dose of DNCB to dilutional patch tests of DNCB applied on day 6. In vivo skin reactivity rank is based on the sum of reactivities of the patch tests. When two or three subjects manifested the same degree of skin reactivity, a mean of the rank values was assigned (e.g., subjects R.C. and E.A. were the 2nd and 3rd least reactive and were each assigned a value of 2.5).

results expressed as the mean counts per minute (± SE) of replicate cultures. The assay system uses a 3-hr incubation with [methyl-^{3}H] thymidine and a Millipore filter collection technique (Millipore Corp., Bedford, Mass.) that has been described elsewhere [12,13].

RESULTS

In general, subjects who displayed a higher degree of cutaneous reactivity to DNCB, as manifested by duration and intensity of dermatitis, showed a greater blastogenic response to DNCB-antigen in vitro. The Figure shows the rank order plot of in vivo skin test reactivity versus the in vitro blastogenic response to DNCB-antigen in 10 DNCB-sensitized subjects. One subject displayed a disproportionately high degree of in vitro blastogenesis to DNCB-antigen compared to his low degree of skin test reactivity. Table I shows in greater detail the in vitro and in vivo results from these sensitized subjects.

Two subjects (R.C. and E.A.) who showed very little skin reactivity also failed to show significant blastogenesis in response to DNCB-antigen. Previous studies from our laboratory on over 30 subjects have shown a similar lack of response in nonsensitized individuals [7]. While subject J.D. has a stimulation ratio of only 2.1, he could be distinguished from nonsensitized controls on the basis of net counts per minute at this time. Furthermore, all 3 of these subjects showed significant blastogenesis to DNCB-antigen following subsequent rechallenge with DNCB.

These 10 subjects as well as 2 additional DNCB-sensitized subjects were studied for lymphokine production following a primary sensitization with DNCB, and 7 of these 12 were also studied following additional rechallenges with DNCB. Very little or no lymphokine activity was detected in any of the 12 subjects during a primary sensitization (Table II, column 1 and footnote *c*). However, significant levels were detected in 2 of 6 subjects studied after one rechallenge with DNCB (Table II, column 2) and 4 of 7 subjects studied after a second rechallenge (Table II, column 3). All but 2 of the 12 subjects, who failed to show significant lymphokine activity following a primary sensitization with DNCB, showed good blastogenic activity to DNCB-antigen with one response in excess of 70,000 cpm (Table I). After the first and second rechallenge, all subjects showed a good blastogenic response to DNCB-antigen. Thus, even in the face of a good blastogenic response in primary cultures, lymphokine activity could be detected only after one or more rechallenges with DNCB.

DISCUSSION

Our results indicate a quantitative correlation between the intensity and duration of skin tests with topical DNCB in vivo and the degree of lymphocyte transformation induced by DNCB-antigen in vitro. The correlation was not invariant. One subject with a very low degree of in vivo skin test reactivity showed a high degree of lymphocyte transformation in vitro. The lack of perfect correlation might be explained by difficulties in accurately quantifying and comparing responses of the in vitro system and/or by difficulties in accurately comparing quantitative in vivo patch-testing results. Alternatively, the occasional disociation may indicate that the in vitro response reflects an earlier or different portion of the immune response than the in vivo skin test. We have previously demonstrated a temporal association between onset of skin reactivity to DNCB and onset of specific in vitro blastogenesis to DNCB-antigen during a primary immune response [6,7]. This, along with the quantitative correlation illustrated

in this paper, suggests that the in vitro blastogenic response to DNCB–antigen, like the skin test, is also a reflection of thymus-dependent CMI. The occasional dissociation, if not due to technical failure, may reflect the measurement of an earlier or different area of thymus-dependent immunity or possibly a measure of two different antigenic determinants, e.g., DNP-skin protein and DNP-erythrocyte protein.

The discovery of lymphokine(s) in allergic contact dermatitis to DNCB further suggests that the in vitro blastogenic response to DNCB–antigen is a manifestation of cellular immunity. Dumonde et al [10] suggested the term lymphokine to describe "non-antibody mediators of cellular immunity generated by lymphocyte activation." The demonstration of lymphokine activity in response to PPD [10], Concanavalin A [14], mixed leukocyte activity [15], and in allergic contact sensitivity to DNCB [8,9] certainly suggests a relationship of these factors to cell-mediated immunity. While some qualitative differences may exist between the various mediators responsible for tuberculin type hypersensitivity, allograft rejection, and allergic con-

TABLE II. *Initiation of lymphokine production following DNCB rechallenge*

Subject	Thymidine incorporation	Primary sensitization	First rechallenge[a]	Second rechallenge[a]
T.J.	Unstimulated	769 ± 509	669 ± 489	270 ± 39
	Stimulated	1416 ± 290	10364 ± 6573	12512 ± 1608
	Lymphokine activity[b]	647	9695	12242
A.L.	Unstimulated	268	156 ± 26	353 ± 1
	Stimulated	1042	618 ± 188	4324 ± 414
	Lymphokine activity	774	462	3971
D.T.	Unstimulated	687 ± 145	1007 ± 426	518 ± 64
	Stimulated	854 ± 288	969 ± 674	576 ± 130
	Lymphokine activity	167	−38	58
R.F.	Unstimulated	1447 ± 735	689 ± 256	305 ± 49
	Stimulated	1332	1109 ± 84	1006 ± 48
	Lymphokine activity	−125	412	701
J.D.	Unstimulated	650 ± 8	758 ± 70	1587 ± 417
	Stimulated	2070 ± 306	3020 ± 258	23506 ± 454
	Lymphokine activity	1420	2262	21919
R.C.	Unstimulated	375 ± 25	776 ± 162	196 ± 12
	Stimulated	1076 ± 186	1061 ± 153	266 ± 12
	Lymphokine activity	701	285	70
B.M.	Unstimulated	128 ± 38	Blood not drawn	191 ± 18
	Stimulated	402 ± 31		3082 ± 12
	Lymphokine activity	274		2891
Average lymphokine activity		551[c]	2180	5979

[a] Subjects were rechallenged with DNCB patch test 2 months (1st rechallenge) and 5 months (2nd rechallenge) after the primary sensitizing application of DNCB.

[b] Primary cultures for lymphokine production were prepared from leukocytes obtained during the third week following sensitization or rechallenge. Supernatants from these primary cultures were harvested after 48 hr of culture; unstimulated and stimulated supernatants constituted 90% of the secondary "insensitive" leukocyte cultures by volume. Lymphokine activity = cpm in secondary stimulated-unstimulated cultures.

[c] Five additional subjects, not included in this table, were studied during a primary sensitization in the same manner. Average lymphokine activity for these 5 subjects was 504 cpm, thus being similar to the 7 subjects shown above.

tact dermatitis, each of these forms of cellular immunity exhibits similar blastogenic lymphokine activity in vitro. The ability to control exposure and thus the degree of sensitivity [16,17] to the primary immunogen, DNCB, has allowed us to discover the absence of significant lymphokine production during a primary immune response and its appearance following subsequent rechallenge with DNCB in humans. While not all subjects show detectable lymphokine activity, even with high levels of blastogenesis to DNCB-antigen, those who demonstrate this activity have been challenged one or more times or have been receiving topical DNCB over an extended period of time as part of an immunotherapy protocol [8].

The in vivo biologic significance of these in vitro defined substances remains speculative. However, the ability of lymphokine(s) to activate lymphocytes from nonsensitized subjects and the absence of significant lymphokine production during a primary immune response suggests the possibility that these substances may represent a mechanism for amplifying the cellular immune response by recruiting previously uncommitted lymphocytes in sensitized subjects undergoing repeated challenge with DNCB. While conclusions on the role of these in vitro defined substances requires further characterization, isolation, and study in both animal and human systems, contact sensitivity with DNCB offers a versatile and ethically feasible system for elucidating the in vivo role of lymphokines in humans.

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