Lymphocyte Transformation during Dinitrochlorobenzene Contact Sensitization

AN IN VITRO AND IN VIVO EVALUATION OF THE PRIMARY IMMUNE RESPONSE IN MAN

A. EDGAR MILLER and WILLIAM R. LEVIS

From the Dermatology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT An evaluation of cell-mediated immunity in man is described that combines the advantages of an in vitro technique, lymphocyte transformation, with the use of contact sensitization to a primary immunogen, dinitrochlorobenzene (DNCB). DNCB, when coupled to autologous or allogeneic peripheral blood leukocytes, forms a complex, DNCB-antigen, that induces lymphocyte transformation specifically in leukocyte cultures from subjects sensitized to DNCB. Sequential studies of lymphocyte transformation to DNCB-antigen show that specifically reactive lymphocytes are first detected at about 10 days after in vivo application of a sensitizing dose of DNCB and reach a peak at about 14–21 days.

INTRODUCTION

Cell-mediated immunity (CMI),[1] a function of the thymus-dependent system, plays an important role in the pathogenesis of many infectious, inflammatory, and neoplastic diseases of man, as well as a central role in transplantation immunity, allergic contact dermatitis, and some immunodeficiency diseases (1, 2). CMI has traditionally been clinically evaluated with tuberculin-type skin tests to various microbial extracts, without knowledge or control over prior exposure. More recently, dinitrochlorobenzene (DNCB)-induced allergic contact dermatitis has been used to measure CMI in many diseases including lymphoid (3, 4) and nonlymphoid malignancies (5, 6). DNCB offers an advantage over microbial antigens in evaluating CMI because natural exposure to DNCB does not occur, and initial application evokes a true primary sensitization (7).

In recent years, the technique of lymphocyte transformation (8, 9) has made it possible to evaluate an immue response to tuberculin and other microbial antigens in vitro. Studies in man and animals indicate that lymphocyte transformation to specific antigens is often an in vitro reflection of CMI (10–12). Attempts to induce lymphocyte transformation with free DNCB have been generally unsuccessful because of its insolubility and toxicity, and the possible absence of a suitable carrier protein in the in vitro system. However, Geczy and Baumgarten (13), using dinitrofluorobenzene, were able to induce specific transformation in lymphocyte cultures from guinea pigs sensitized by the injection of DNCB.

Our studies show that lymphocyte transformation and DNA synthesis can occur in response to an antigen prepared by coupling DNCB to peripheral blood leukocytes (DNCB-antigen). The lymphocyte transformation to DNCB-antigen occurred only in subjects sensitized to DNCB. The specificity of the lymphocyte transformation and correlation with the onset of allergic contact dermatitis are demonstrated by sequential changes in in vitro lymphocyte transformation to DNCB-antigen during a primary in vivo sensitization with DNCB.

METHODS

Sensitization to DNCB

A sensitizing dose of 2,000 μg DNCB in 0.1 ml acetone was applied to the skin of the medial aspect of the upper arm within a 2-cm diameter polyethylene ring, allowed to evaporate, and covered by an adhesive bandage for 1 wk (4, 5).

Received for publication 26 December 1972 and in revised form 9 April 1973.

[1] *Abbreviations used in this paper:* CMI, cell-mediated immunity; DMSO, dimethyl sulfoxide; DNCB, dinitrochlorobenzene; PHA, phytohemagglutinin.

Subjects

Sensitized patients. One group of subjects consisted of three patients with multiple basal cell carcinomas. This group was sensitized as above, patch tested with different concentrations of DNCB, and treated daily with topical DNCB for prolonged periods of time as part of an immunotherapy protocol. 48-h occlusive patch tests (Johnson & Johnson Sheer Patches, Johnson & Johnson, New Brunswick, N. J.) produced erythema and induration in all three patients at 1 μg DNCB.

Sensitized volunteers. A second group of subjects consisted of two healthy adult volunteers. These subjects received sensitizing doses of DNCB on both arms on the same day, but did not receive additional topical DNCB. Sensitization in this group was determined by a spontaneous flare of dermatitis within the sensitization site which occurred during the 2nd wk after sensitization (5).

"Insensitive" subjects. This group consisted of five healthy blood donors with no history of exposure to DNCB.

Obtaining mononuclear leukocytes for culture and as a source for DNCB-antigen

Peripheral leukocytes were obtained by a centrifugation technique which yields 72–92% lymphocytes and is described in detail elsewhere (14). Briefly, heparinized venous blood was centrifuged at approximately 300 *g* for 5 min. The resulting leukocyte-rich plasma was removed with a Pasteur pipette and utilized in the preparation of leukocyte cultures, used for the preparation of DNCB-antigen (see below), or stored frozen by a modification of techniques described by Chess, Bock, and Mardiney (15) that maintains the in vitro blastogenic response to specific antigens and phytohemagglutinin (PHA) at a level indistinguishable from fresh cells for at least 8 mo.[2]

Preparation of DNCB-antigen

4–6 ml of mononuclear leukocyte-rich plasma, obtained by centrifugation as outlined above, was placed in a 10-cm^3 conical tube and centrifuged at 150 *g* for 6 min. The plasma was then decanted, and the remaining cell pellet was suspended in medium 199 with a Pasteur pipette and centrifuged at 150 *g* for 6 min. The supernate was decanted and the pellet then suspended in 10 cm^3 of undiluted DMSO (dimethyl sulfoxide, Aldrich Chemical Co., Inc., Milwaukee, Wis) containing 1 g/100 ml DNCB (1-chloro-2,4-dinitrobenzene, Eastman Kodak Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.). After a 1-h incubation at 38°C the tube was centrifuged at 150 *g* for 6 min, the DNCB-DMSO supernate decanted, and the cell pellet washed with three 10-cm^3 aliquots of medium 199, each utilizing a 6-min centrifugation at 150 *g*. Some pellets were washed once with DMSO followed by two washes with medium 199. The cell pellets resulting from this procedure are referred to as DNCB-antigen. When DNCB-antigen is used in this wet form, the amount to be added to leukocyte cultures is quantitated by counting the number of intact leukocytes in a hemocytometer. When the DNCB-antigen was stored in a lyophilized form, the amount to be added to leukocyte cultures was determined gravimetrically. For lyophilization, DNCB-antigen was suspended in sterile water in a glass vial, frozen in ethanol and dry ice, and placed overnight on a VirTis lyophilizer (VirTis Co., Inc., Gardiner, N. Y.). The resultant powder was stored in the dark at 4°C. DNCB-antigen was prepared from two patients with chronic lymphocytic leukemia (peripheral leukocyte counts of 250,000 and 600,000/mm^3) by sedimenting heparinized venous blood at 38°C for 30 min and treating the leukocyte-rich plasma in the same manner as the mononuclear rich plasma obtained by centrifugation.

[2] Unpublished data of the authors.

Preparation of leukocyte cultures

"Mononuclear leukocyte culture fluid" was prepared by diluting the mononuclear leukocyte-rich plasma obtained by centrifugation with four parts of tissue culture medium 199 containing penicillin and streptomycin. This method yields a final concentration of 0.4—2×10^6 cells/ml of which 72–92% are lymphocytes (14).

Addition of DNCB-antigen

Either wet or lyophilized DNCB-antigen was suspended in cell-free culture fluid (20% plasma in medium 199) and added to an equal volume of mononuclear leukocyte culture fluid. Leukocyte cultures without DNCB-antigen were prepared by diluting mononuclear leukocyte culture fluid with an equal volume of cell-free culture to maintain the same number of leukocytes in control cultures and DNCB-antigen-stimulated cultures. Replicate cultures with and without DNCB-antigen were also stimulated with PHA (Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.) at a final concentration of 2.0–2.5 μg/ml of culture. When the DNCB-antigen was cultured alone, with or without PHA, it was diluted in cell-free culture fluid so that the concentration was the same as in DNCB-antigen-stimulated cultures containing mononuclear leukocyte culture fluid. The plasma was the same for all stimulated and unstimulated cultures within each experiment. All cultures were cultured in 0.5-ml vol at 38°C with room air as the gas phase in 12×35 mm screw-capped vials (Arthur H. Thomas Co., Philadelphia, Pa.).

Evaluation of cultures

Tritiated thymidine incorporation during DNA synthesis was measured between the 40th and 86th h in PHA-stimulated cultures and between the 90th and 132nd h in cultures without PHA. In each experiment, duplicate or triplicate cultures were assayed at the same time and the results expressed as the mean counts per minute (±SE) of replicate cultures. The assay system uses a 3-h incubation with [*methyl*-^{3}H]thymidine and a Millipore filter collection technique (Millipore Corp., Bedford, Mass.) that has been described elsewhere (16, 17). Morphologic confirmation of blastogenesis was obtained by light microscopy of Giemsa-stained smears of replicate cultures.

RESULTS

DNCB-antigen, prepared by incubating autologous leukocytes with varying concentrations of DNCB in DMSO, induced varying degrees of lymphocyte transformation and DNA synthesis in leukocyte cultures from a patient sensitized to DNCB. DNA synthesis increased from a mean of 20,000 cpm with 0.001% DNCB to over 60,000 cpm when 1% DNCB was used in the preparation of DNCB-antigen. Autologous leukocytes incubated in DMSO without DNCB failed to induce transformation

Table I
Lymphocyte Transformation to DNCB-Antigen Prepared with Varying Concentrations of DNCB

DCNB-antigen‡	DNA Synthesis in cpm*	
	DCNB-antigen§ + PHA	DNCB-antigen + leukocytes‖
1 g/100 ml DNCB in DMSO	38±2	68,635±44,794
0.1 g/100 ml DNCB in DMSO	32±8	47,010±22,942
0.01 g/100 ml DNCB in DMSO	33±5	22,237±8,850
0.001 g/100 ml DNCB in DMSO	46±14	20,562±1,683
0% DNCB in DMSO	49±15¶	427±131
None		649±243

* Values for tritiated-thymidine incorporation are expressed as mean ±SE of counts per minute obtained from replicate 0.5-ml cultures assayed on the 5th day; PHA-stimulated cultures were assayed on the 2nd day.
‡ Incubation period with DNCB-DMSO was 1 hr.
§ The concentration of DNCB-antigen was constant at 0.25 × 10^6 DNCB-treated autologous leukocytes/ml of culture.
‖ The concentration of leukocytes was constant at 0.5 × 10^6/ml of culture; the source of leukocytes was a patient who was patch test-sensitive to non-irritating concentrations of DNCB.
¶ Aliquots of leukocytes used in the preparation of DNCB-antigen, when suspended in medium 199 rather than DMSO and stimulated with PHA, gave 134,313±25,217 cpm when assayed on the 2nd day.

(Table I, right column). Leukocytes used in the preparation of DNCB-antigen (see Methods) were killed when incubated in DMSO for 1 h at 38°C, as evidenced by a lack of response to PHA when cultured alone in this experiment (Table I) and in other experiments.

Lymphocyte transformation, as measured by DNA synthesis, increased with increasing concentrations of stored lyophilized DNCB-antigens up to an average of 27,000 cpm at 40 μg/ml (Fig. 1). DNA synthesis was less at 200 μg/ml.

DNCB-antigen, prepared with allogeneic insensitive leukocytes from healthy and leukemic subjects, induced lymphocyte transformation and DNA synthesis in leukocyte cultures from DNCB-sensitized subjects (Table II) but not in insensitive subjects (see below). Thus, DNCB-antigen, capable of stimulating sensitive leukocytes, can be prepared from allogeneic insensitive leukocytes as well as from autologous sensitive leukocytes. Furthermore, preliminary studies have shown that some tissue culture cells (e.g. HeLa) and red blood cells can also be used to prepare DNCB-antigen.[3]

Leukocyte cultures from subjects insensitive to DNCB failed to respond to DNCB-antigen prepared from either allogeneic or autologous leukocytes (Table III). The inability of insensitive leukocytes to respond to DNCB-antigen was specific because, in the presence of DNCB-antigen, PHA induced responses ranging from 80,000 to 255,000 cpm (Table III). The DNCB-antigens used in the negative leukocyte cultures were active, as evidenced by their simultaneous ability to stimulate leukocytes from

[3] Unpublished data of the authors.

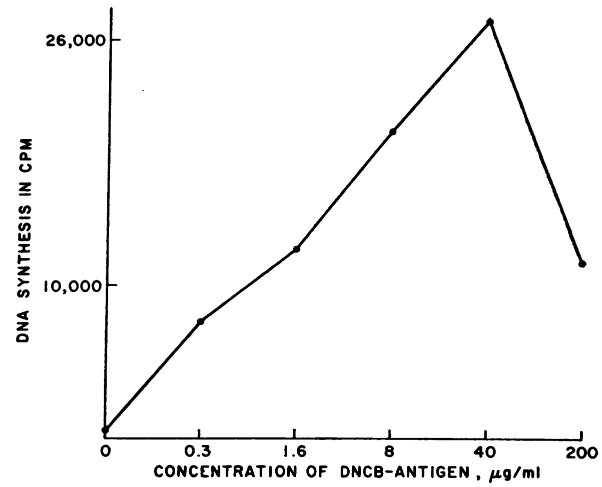

Figure 1 Lymphocyte transformation at varying concentrations of DNCB-antigen. Values for tritiated thymidine incorporation are expressed as mean counts per minute of triplicate 0.5-ml cultures assayed on the 4th day. The concentration of sensitive leukocytes was constant at 0.8 × 10^6/ml of culture. DNCB-antigen was prepared from autologous leukocytes and 1% DNCB in DMSO and was utilized in the lyophilized state.

Table II
Ability of DNCB-Antigen Prepared from Allogeneic DNCB-Insensitive Leukocytes to Stimulate DNCB-Sensitive Leukocytes

Allogeneic DNCB-antigen‡	DNA synthesis in cpm*		Stimulation ratio‖
	Sensitive leukocytes§ alone	DNCB-antigen + sensitive leukocytes	
1	2,134±186	47,571±7,209	22
2	842±118	11,523±2,277	14
3	2,750±416	81,833±5,631	30
4	2,750±416	33,448±8,092	12
5	2,602±898	15,137±11,217	6
6	2,602±898	72,516±1,854	28
7	2,602±898	25,516±6,441	10
8	1,688±268	48,875±1,615	29
9	300±46	16,241±7,381	54

* Values for tritiated thymidine incorporation are expressed as mean (±SE) of counts per minute obtained from replicate 0.5-ml cultures assayed on the 4th or 5th day.
‡ Antigens 1–7 were prepared from seven normal volunteers and 8 and 9 from two patients with chronic lymphocytic leukemia; the concentration of lyophilized DNCB-antigen was constant in each experiment (range 25–50 μg/ml of culture).
§ The concentration of leukocytes was constant in each experiment (range 0.5–1.3 × 10^6/ml of culture).
‖ Stimulation ratio $= \frac{\text{leukocytes + DNCB-antigen (cpm)}}{\text{leukocytes (cpm)}}$.

Table III
Specificity of Lymphocyte Transformation to DNCB-Antigen

	DNA synthesis in cpm*			DNA synthesis in cpm			
Exp.	Sensitive leukocytes‡	Sensitive leukocytes DNCB-antigen§	Stimulation ratio‖	Insensitive leukocytes	Insensitive leukocytes + DNCB-antigen	Insensitive leukocytes + DNCB-antigen + PHA	Stimulation ratio
1	990±442	12,265±9	12	395±27	533±265	147,189±765	1.3
2	318±198	3,167±335	10	573±61	337±159	237,098±9,665	0.6
3	2,134±186	47,571±7,209	22	4,368±818	3,532±652	83,177±11,055	0.8
4	1,089±23	27,666±12	25	763±85	753±213	83,859±10,273	1.0

* Values for tritiated-thymidine incorporation are expressed as mean ±SE of counts per minute obtained from replicate 0.5-ml cultures assayed on the 4th or 5th day; PHA-stimulated cultures were assayed on the 3rd day.
‡ The concentration of leukocytes was constant for each experiment (range 0.44–1.3 × 10^6/ml of culture). Experiment 1 utilized frozen-stored leukocytes collected before and after sensitization to DNCB.
§ DNCB-antigen was prepared from autologous peripheral leukocytes in experiment 1, and from allogeneic in experiments 2, 3, and 4; experiment 4 utilized DNCB-antigen prepared from chronic lymphocytic leukemia leukocytes; the concentration of DNCB-antigen was constant in each experiment.
‖ Stimulation ratio = (leukocytes + DNCB-antigen [cpm])/leukocytes (cpm).

subjects sensitized to DNCB (Table III). In three of the four paired experiments in Table III (experiments 2, 3, and 4) the DNCB-antigen was prepared using leukocytes allogeneic to both sensitive and insensitive subjects. In each case the DNCB-negative leukocytes specifically failed to respond. These allogeneic DNCB-antigens did not induce mixed leukocyte culture reactivity in DNCB-insensitive leukocyte cultures.

DNCB-antigen induced transformation of sensitive lymphocytes, whether they were suspended in autologous plasma or in allogeneic plasma from a DNCB-insensitive donor. Sensitive leukocytes were washed repeatedly before being suspended in either autologous or allogeneic plasma, and responded to DNCB-antigen with stimulation ratios of 31 and 28, while insensitive leukocytes suspended in plasma from the same sensitive donor simultaneously failed to respond to DNCB-antigen (Table IV).

Table IV
Specificity of Lymphocyte Transformation to DNCB-Antigen in the Presence of Plasma from Sensitive and Insensitive Subjects

		DNA Synthesis in cpm*		
Leukocytes‡	Plasma	Leukocytes	Leukocytes + DNCB-antigen§	Stimulation ratio
Sensitive	Sensitive	219±27	6,781±970	31
Sensitive	Insensitive	845±169	23,925±1,108	28
Insensitive	Sensitive	480±123	349±33‖	0.7

* Values for tritiated thymidine incorporation are expressed as mean ±SE of counts per minute obtained from triplicate 0.5-ml cultures on the 4th day.
‡ Concentration of leukocytes was constant at 0.5 × 10^6/ml of culture. Leukocytes were obtained from a sensitive patient and an insensitive volunteer.
§ DNCB-antigen was prepared from chronic lymphocytic leukemia leukocytes and concentration was 5 μg/ml of culture.
‖ Tetanus toxoid added to replicate cultures of insensitive leukocytes in the presence of DNCB-antigen induced DNA-synthesis (56,056±6,544 cpm) demonstrating that these cultures specifically failed to respond to DNCB-antigen.

The degree of lymphocyte transformation to DNCB-antigen is shown in leukocytes obtained from a volunteer before, during, and after in vivo challenge with DNCB (Fig. 2). Leukocytes were obtained on day 0 and

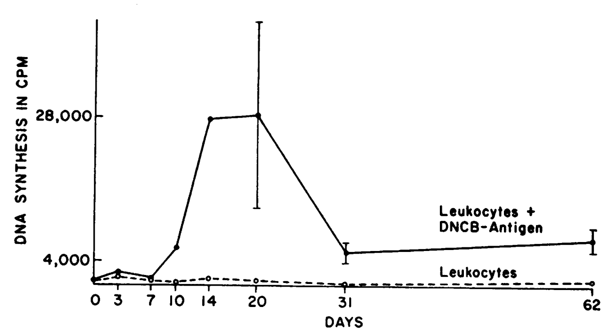

Figure 2 Lymphocyte transformation to DNCB-antigen during primary sensitization to DNCB. Values for tritiated thymidine incorporation are expressed as mean counts per minute of duplicate 0.5-ml cultures assayed on the 4th day; vertical bars represent ±SE when SE doesn't fall within the "point." Volunteer received topical application of DNCB on day 0, leukocytes were collected and stored frozen on days 0, 3, 7, 10, 14, 20, 31, and 62, and the experiment was performed on day 90. Concentration of leukocytes was kept constant at 0.65 × 10^6/ml of culture. DNCB-antigen was prepared from allogeneic leukemia lymphocytes and utilized at 10 μg/ml of culture. PHA-stimulated cultures of leukocytes from day 0, 3, and 7 in the presence of DNCB-antigen responded with DNA synthesis of 74,000–102,000 cpm.

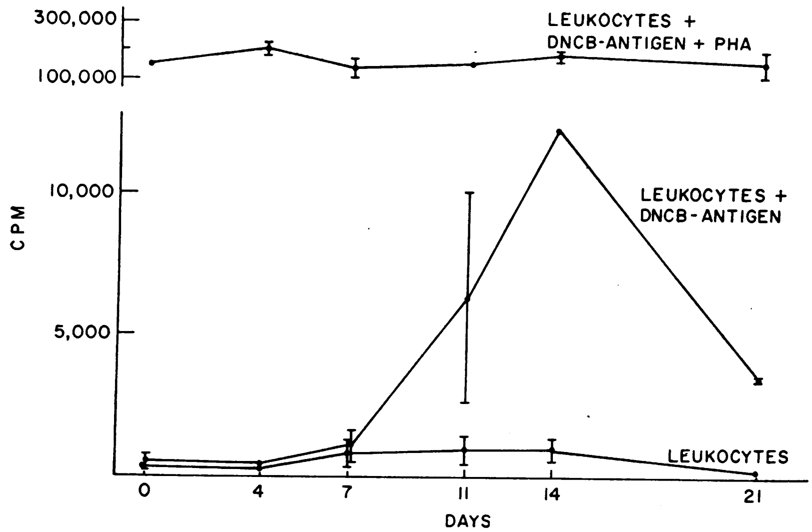

FIGURE 3 Lymphocyte transformation to DNCB-antigen during primary sensitization to DNCB. Counts per minute (±SE) of duplicate cultures assayed on the 5th day for leukocytes cultured alone and with DNCB-antigen, and on the 3rd day when stimulated with PHA. Leukocytes obtained on days 0, 4, 7, 11, 14, and 21 after primary sensitization, stored frozen, and cultured at a concentration of 0.9×10^6/ml of culture alone, with DNCB-antigen, and with PHA in the presence of DNCB-antigen. DNCB-antigen was prepared from autologous leukocytes and utilized at 100,000 cells/ml of culture.

a sensitizing dose of DNCB subsequently applied. Leukocytes were again obtained from this volunteer on days 3, 7, 10, 14, 20, 31, and 62 after the sensitizing application of DNCB. All leukocytes were stored frozen, and on day 90 leukocyte cultures from each of these days were prepared with and without DNCB-antigen. DNA synthesis was measured 4 days later by [H^3]thymidine incorporation. Leukocytes obtained on days 0, 3, and 7 failed to respond to DNCB-antigen. The inability to respond was specific for DNCB-antigen, because the addition of PHA to these leukocyte cultures gave 74,000–102,000 cpm in the presence of DNCB-antigen. DNCB-antigen induced lymphocyte transformation in leukocyte cultures from days 10, 14, 20, 31, and 62 with the highest responses in cultures from days 14 and 20. The in vitro conversion of lymphocyte transformation to DNCB-antigen on day 10 correlated with an in vivo flare of contact dermatitis at the sensitizing site, which occurred between days 10 and 14.

Sequential studies using frozen-stored leukocytes from a second volunteer undergoing DNCB sensitization are shown in Fig. 3, using DNCB-antigen prepared from autologous leukocytes. Leukocytes obtained on days 0, 4, and 7 failed to respond to DNCB-antigen. The inability to respond was specific for DNCB-antigen, because the addition of PHA to these leukocyte cultures gave 140,-000–210,000 cpm in the presence of DNCB-antigen. DNCB-antigen induced lymphocyte transformation in leukocyte cultures from days 11, 14, and 21, with the highest responses on days 11 and 14. The in vitro conversion of lymphocyte transformation to DNCB-antigen, first detected on day 11, correlated with an in vivo "flare" of the primary sensitizing site, which occurred on day 9.

DISCUSSION

There were several problems in adapting DNCB to the lymphocyte transformation technique. DNCB, like many common contact sensitizers, is highly water-insoluble, in contrast to PPD and other soluble microbial antigens, which have been successfully used to induce lymphocyte transformation. Geczy and Baumgarten (13), by preheating DNFB in a phosphate buffer at 60°C, were able to induce transformation of lymphocytes from guinea pigs sensitized by the injection of DNCB. They repeated incubations and washing procedures with each experiment, probably to avoid toxicity from free DNFB. DNCB in its unconjugated insoluble state is also highly toxic in leukocyte cultures.

Our results show that DNCB coupled to leukocytes in a DMSO solvent system forms a complex that behaves as an antigen (DNCB-antigen). We have not yet determined the chemical nature or mechanism of action, but DNCB-antigen is particulate, storable, has a low degree of toxicity, and induces lymphocyte transformation only in leukocyte cultures from subjects sensitized to DNCB.

The specificity of lymphocyte transformation to DNCB-antigen is dependent on the cells, because sensitized leukocytes respond in cultures containing plasma from insensitive subjects, and insensitive leukocytes fail to respond in cultures containing plasma from sensitized subjects. Although lymphocyte transformation to DNCB-antigen requires responding leukocytes from a sensitized subject, the DNCB-antigen can be prepared using allogeneic insensitive leukocytes from healthy or leukemic subjects. Sequential studies of lymphocyte transformation to DNCB-antigen show that specifically reactive lymphocytes are first detected about 10 days after in vivo topical application of a sensitizing dose of DNCB, and reach a peak at about 14–21 days. Thereafter the degree of lymphocyte transformation to DNCB-antigen levels off but is still detectable for at least 2 mo after sensitization.

The addition of an in vitro method expands the usefulness of DNCB for the evaluation and study of CMI. A variety of antigen-released mediators of cellular immunity have been described since the advent of in vitro techniques (18, 19), and the DNCB system offers a controlled, systematic approach for the production, characterization, and study of these antigen-released mediators in man. In humans, the degree of sensitization to DNCB can be controlled, to include a state of hyporesponsiveness or tolerance (20). The addition of an in vitro technique may allow further understanding of the tolerant state. In addition to using DNCB for evaluating CMI in patients with malignancies, there have been several successful therapeutic attempts using DNCB in the treatment of malignancies, both primary and metastatic to the skin (21–23). Lymphocyte transformation can be used for in vitro monitoring during DNCB immunotherapy, and as a possible approach for further understanding of the mechanisms involved. The epicutaneous application of a small amount of a suspected allergen is the classical diagnostic procedure for allergic contact dermatitis, but the inherent risk of sensitization should not be underestimated. In vitro approaches may provide diagnostic tests free from the risk of inducing or increasing sensitization.

ACKNOWLEDGMENTS

The authors thank Dr. Jay H. Robbins and Dr. Joost J. Oppenheim for their helpful comments and criticisms.

REFERENCES

1. Samter, M. 1971. Immunological Diseases. Little, Brown & Co., Inc., Boston, Mass.
2. Waksman, B. H. 1972. Measurement of cell-mediated immunity. *N. Engl. J. Med.* **286**: 431.
3. Aisenberg, A. C. 1962. Studies on delayed hypersensitivity in Hodgkin's disease. *J. Clin. Invest.* **41**: 1964.
4. Brown, R. S., H. A. Haynes, H. T. Foley, H. A. Godwin, C. W. Berard, and P. P. Carbone. 1967. Hodgkin's disease immunologic, clinical and histologic features in 50 untreated patients. *Ann. Intern. Med.* **67**: 291.
5. Catalona, W. J., P. T. Taylor, A. S. Rabson, and P. B. Chretien. 1972. A method for dinitrochlorobenzene contact sensitization *N. Engl. J. Med.* **286**: 399.
6. Eilber, F. R., and D. L. Morton. Impaired immunologic reactivity and recurrence following cancer surgery. *Cancer.* **25**: 362.
7. Kligman, A. M., and W. L. Epstein. 1959. Some factors affecting contact sensitization in man. *In* Mechanisms of Hypersensitivity. J. H. Shaffer, G. A. LoGrippo, and M. W. Chase, editors. Little, Brown & Co., Boston, Mass. 713.
8. Ling, N. R. 1968. Lymphocyte Stimulation. North-Holland Publishing Co., Amsterdam.
9. Robbins, J. H. 1964. Tissue culture studies of the human lymphocyte. *Science (Wash. D. C.).* **146**: 1648.
10. Oppenheim, J. J. 1968. Relaitonship of *in vitro* lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. *Fed. Proc.* **27**: 21.
11. Mills, J. A. 1966. The immunologic significance of antigen induced lymphocyte transformation in vitro. *J. Immunol.* **97**: 239.
12. Zweiman, B. 1967. Temporal relationship between tuberculin skin reactivity and in vitro mitotic response. *Immunology.* **13**: 315.
13. Geczy, A. F., and A. Baumgarten. 1970. Lymphocyte transformation in contact sensitivity. *Immunology.* **19**: 189.
14. Levis, W. R., and J. H. Robbins. 1972. Methods for obtaining purified lymphocytes, glass-adherent mononuclear cells, and a population containing both types from human peripheral blood. *Blood.* **40**: 77.
15. Chess, L., G. N. Bock, and M. R. Mardiney. 1972. Reconstitution of the reactivity of frozen-stored human lymphocytes in the mixed lymphocyte reaction and in response to specific antigen. *In* Proceedings of the Sixth Leukocyte Culture Conference. M. R. Schwarz, editor. Academic Press, Inc., New York. 501.
16. Robbins, J. H., P. G. Burk, and W. R. Levis. 1970. A rapid and sensitive assay for DNA synthesis in leukocyte cultures. *Lancet.* **2**: 98.
17. Robbins, J. H., J. J. Gart, W. R. Levis, and P. G. Burk. 1972. The millipore filter assay technique for measuring tritiated thymidine incorporation into DNA in leukocyte cultures. *Clin. Exp. Immunol.* **11**: 629.
18. Lawrence, H. S., and M. Landy. 1969. Mediators of Cellular Immunity. Academic Press, Inc., New York.
19. Bloom, B. R., and P. R. Glade. 1971. In Vitro Methods in Cell-Mediated Immunity. Academic Press Inc., New York.
20. Lowney, E. D. 1968. Immunologic unresponsiveness to a contact sensitizer in man. *J. Invest. Dermatol.* **51**: 411.
21. Ratner, A. C., D. S. Waldorf, and E. J. Van Scott. 1968. Alterations of lesions of mycosis fungoides lymphoma by direct imposition of delayed hypersensitivity reactions. *Cancer.* **21**: 83.
22. Klein, E. 1969. Hypersensitivity reactions at tumor sites. *Cancer Res.* **29**: 2351.
23. Stjernsward, J., and A. Levin. 1971. Delayed hypersensitivity-induced regression of human neoplasms. *Cancer.* **28**: 628.