Correlation of in Vivo and in Vitro Assays of Immunocompetence in Cancer Patients

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SUMMARY

Of 52 cancer patients studied for their in vitro response in lymphocyte blastogenesis assays, 50 were also studied for immunocompetence by in vivo assays. The in vivo assays used were the delayed cutaneous hypersensitivity reaction to the primary stimulus of 2, 4-dinitrochlorobenzene (DNCB), and the recall reactions to four common microbial antigens. The in vitro assays were the blastogenic response to three mitogens (phytohemagglutinin, pokeweed mitogen, concanavalin A) and the mixed lymphocyte culture (MLC) reaction. The carcinoma patients demonstrated an apparent impairment of skin test reactions, but the least impairment of their lymphocyte blastogenesis reactions. The melanoma patients had notable defects in lymphocyte function tests but less impairment of the skin test reactions. Results for sarcoma patients were intermediate in both in vivo and in vitro assays. When the patients were grouped according to response to DNCB, no significant differences in responses to mitogens between the DNCB reactors and nonreactors were observed. However, the DNCB nonreactors had markedly reduced responses in MLC. Those patients with a poor response in recall antigen skin tests showed a diminished response in MLC and also a reduced response to the mitogens. It is postulated that antigen recognition defects can exist in cancer patients that can be detected by the DNCB or MLC tests. Additionally, there may be lymphocyte proliferation defects demonstrable in patients with certain histopathologies of cancer, especially melanoma, or in those in whom secondary immune responsiveness, as reflected by recall antigen skin tests, is impaired. These data suggest that the mitogen concanavalin A and the MLC are probably more useful screening assays of in vitro immunocompetence than is the more commonly used mitogen, phytohemagglutinin.

INTRODUCTION

An extensive literature indicates that the functional status of the immune system may have a decisive impact on the clinical response of cancer patients to their disease. This appears to be especially true in patients with lymphoproliferative cancers and is most apparent from studies of Hodgkin’s disease and the leukemias. In these diseases, anergy is apparent in responses of the delayed hypersensitivity type, and many patients exhibit defects in in vitro assays of lymphocyte function as well. This is not surprising considering that a malignant transformation of immunocompetent cells would very probably induce aberrations of immune functions.

However, a consistent pattern of immunological deficiency is not as clear in patients with nonlymphoid cancers. Certain histological types of cancer, including sarcomas and squamous carcinomas, have been reported to have depressed responses to the mitogen PHA (3). Other studies have indicated that these types of cancer, and also colon carcinomas and intracranial tumors, are associated with serum factors that inhibit lymphocyte-proliferative reactions (2, 4, 12). Other laboratories have been unable to demonstrate any defects in lymphocyte function (10, 11) in similar studies.

In addition to in vitro assays of lymphocyte function, extensive in vivo tests have been used to assess the immune status of cancer patients. Two types of measurement of the delayed hypersensitivity response have been used: the common skin test antigens, in which the ability to manifest a “recall” delayed hypersensitivity reaction to microbial antigens is assessed; and the primary response to a new agent. The former has the advantage of ease of application, but its interpretation is complicated by the variability of exposure to these antigens in the population. Eilber and Morton (5) assessed immunocompetence of cancer patients by measuring their response to a primary sensitization with a contact allergen, DNCB. Patients who failed to respond to the allergen had a uniformly poor prognosis in that they were either inoperable or had a rapid recurrence following surgical resection of the tumor.

The purpose of our study was to determine the degree of correlation among the various in vivo and in vitro assays that could be used to assess immunocompetence in cancer patients. The tests used were the most commonly used of the in vivo and in vitro assays: (a) response to common

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2 The abbreviations used are: PHA, phytohemagglutinin; DNCB, 2,4-dinitrochlorobenzene; MLC, mixed lymphocyte culture; PWM, pokeweed mitogen; Con A, concanavalin A.
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microbial skin test antigens; (b) the response to a primary sensitization to DNCB; and (c) lymphocyte function tests in vitro based on the blastogenic response to mitogens and in MLC.

MATERIALS AND METHODS

Patients. Each patient tested either had not been previously treated or had a suitable interval (usually 3 months) between any prior therapy and testing. Patients with lymphoproliferative cancers and juveniles with cancer were excluded. Control subjects were healthy volunteers of approximately the same age range as the cancer patients. They had no history of prior malignant disease and were not taking any medication that might affect their immune system.

The majority of patients studied (29 of 52) had malignant melanoma. Also studied were 9 patients with various types of soft-tissue or skeletal sarcomas (3 osteosarcomas, 2 leiomyosarcomas, and 1 each of spindle cell sarcoma, rhabdomyosarcoma, liposarcoma, and synovial cell sarcoma) and 14 patients with adenocarcinoma (8 breast carcinomas, 2 colon carcinomas, 1 lung carcinoma, and 3 undifferentiated carcinomas of unknown primary sites). Four patients (2 melanomas and 2 sarcomas) had no evidence of metastatic disease. Sixteen patients (15 melanomas and 1 sarcoma) had disease that was considered regional, and the remaining 32 patients had distant metastases. All of the 14 sarcoma patients, 6 of the 9 sarcoma patients, and 12 of the 29 melanoma patients had distant disease. The carcinoma patients also tended to be older (average age of 52.9) than the other groups (melanomas, 40.1; sarcomas, 36.0).

Lymphocytes were recovered from heparinized blood by density-gradient centrifugation on Ficoll-Isopaque gradients (1). The lymphocytes were washed 3 times in buffered 0.9% NaCl solution, counted, and resuspended in Roswell Park Memorial Institute Medium 1640 containing 20% human AB serum, antibiotics, and 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer. The purification technique routinely yielded lymphocyte samples with less than 5% granulocyte contamination. The numbers of monocytes varied, comprising 5 to 15% of the cells recovered from both controls and cancer patients. The only batch of human AB serum used had been tested with several lymphocyte samples from normal donors and had allowed lymphocyte proliferation equivalent to the autologous serum of those donors.

Blastogenesis Assays. Lymphocyte blastogenesis was assessed by the microassay of Sengar and Terasaki (13). Lymphocyte samples of 5 x 10^6 cells were tested in quadruplicate in Beckman microtubes (Beckman Instruments, Inc., Fullerton, Calif.) for each stimulator used. The mitogens used were: PHA (Wellcome, Beckenham, England), 0.5% final concentration; PWM (Grand Island Biological Co., Grand Island, N. Y.), 0.5%; and Con A (Miles Laboratories, Kankakee, Ill.), 5 µg/culture. In the MLC test, each lymphocyte sample to be tested was placed in culture with a standard pool of allogeneic lymphocytes prepared from 16 normal donors. Then the sample was aliquoted and stored by a viable freezing technique similar to the method of Mangi and Mardinay (8). These lymphocytes, referred to as stimulator cells, were thawed as needed; washed; treated with mitomycin C, 50 µg/ml (Nutritional Biochemicals Corp., Cleveland, Ohio), for 30 min at 37°; and washed 3 times. Then 5 x 10^6 cells were added to the responder lymphocytes. Unstimulated lymphocytes, lymphocytes to which mitomycin-treated autologous lymphocytes were added, stimulators (mitogen- or mitomycin-treated cells) alone, medium alone, and double mitomycin controls, consisting of mitomycin-treated responder and stimulator cells, were used as controls of the technique.

The tubes containing the lymphocytes plus mitogen or stimulator cells were incubated for 5 days, pulsed with 0.5 µCi of thymidine-3H (New England Nuclear, Boston, Mass.), and harvested after 18 hr of labeling. This time period was chosen because it was the optimum in the MLC assay. Harvesting was performed by precipitation in trichloroacetic acid. Scintillation counting of the trichloroacetic acid precipitate was performed as described by Sengar and Terasaki (13). Data were expressed as net cpm of incorporated label. Net cpm were calculated from the average cpm of the stimulated lymphocytes minus the average cpm of the unstimulated lymphocytes minus the average cpm of the stimulators alone. Net cpm were used rather than a ratio of cpm of stimulated lymphocytes to unstimulated lymphocytes, because we found that the background cpm of the unstimulated lymphocytes were much more variable upon repeated testing of the same sample than were the cpm of mitogen-stimulated lymphocytes (S. H. Golub, H. L. Sulit, and D. L. Morton, in preparation). Comparison of the background cpm of patients with those of controls did not show any significant differences.

Skin Testing. Patients were sensitized with 2000 µg DNCB using the method of Eilber and Morton (5). The patients were challenged with 100 µg of DNCB on the day of sensitization and with doses of 100, 50, and 25 µg 14 days postsensitization. After an additional 48 hr, the skin tests were examined and graded. Each dose was graded as 1+ (erythema and induration of less than 5 mm), 2+ (induration between 5 and 10 mm), 3+ (induration of greater than 10 mm), or 4+ (bullae formation). Patients were considered “responsive” if they fulfilled any of the following criteria: (a) a 2+ response to the memory dose applied at the time of sensitization; (b) a 1+ or 2+ response to the 25- or 50-µg doses (with a 2+ or stronger reaction at 50 or 100 µg); (c) a 3+ response at the 100 µg site only.

At the time of DNCB challenge, each patient was also tested for delayed cutaneous hypersensitivity to 4 common microbial recall antigens. The antigens used were: mumps, 2 units (Eli Lilly and Co., Indianapolis, Ind.); purified protein derivative, 5 units, intermediate strength (Connaught, Toronto, Canada); Varidase, 10 units (Lederle Laboratories, Pearl River, N. Y.); and Monilia antigen, 2 units (Hollister-Stier Laboratories, Burbank, Calif.). All antigens were applied as 0.1-ml intradermal injections in the forearm, contralateral to the DNCB test.
A response of greater than 5 mm of induration was considered positive.

RESULTS

Skin Test Results. Results of the skin tests are summarized in Table 1. DNCB nonreactors constituted 28% of the tested patients and were found in all histological groups. There was a higher proportion of DNCB nonreactors among the carcinoma patients (7 of 14, or 50%) than in the melanoma patients (5 of 28, 18%), or sarcoma patients (2 of 8, 25%). Similarly, 28% of the tested patients who exhibited no or a single positive skin test to the 4 recall antigens were found more often in the carcinoma group (6 of 14, 43%) than in the melanoma group (4 of 28, 14%), or the sarcoma group (2 of 8, 25%). Although the carcinoma group appeared to have a more pronounced immunological impairment as measured by cutaneous hypersensitivity, these results may relate to age or degree of tumor burden rather than to histological type of tumor. The DNCB nonreactors, as well as the recall antigen nonreactors, were frequently among the older patients, as reflected by the greater average age (Table 1). On the other hand, those patients exhibiting 3 or 4 positive recall tests also had a greater average age than those demonstrating 2 positive tests, which probably reflects a greater exposure to the common antigens.

Response According to Histological Type of Disease in Lymphocyte Function Tests. The average net cpm in each of the blastogenesis assays for the various histological groups are summarized in Table 2. The patients with melanoma had decreased levels of blastogenesis in response to mitogens compared to the other patients. All groups displayed decreased blastogenesis in response to Con A, but the melanoma group was most significantly reduced. Only the melanoma group exhibited impairment in response to PHA, although this small reduction was significant at only the 0.05 level by the Mann-Whitney test. The response of melanoma patients to allogeneic lymphocytes in the MLC test also was reduced, although this was not statistically significant.

The sarcoma patients had normal responses to the mitogen PHA and statistically insignificant reductions in response to PWM and in MLC. The sarcoma patients were less responsive to Con A than were the controls, but they were more responsive than the melanoma group. The carcinoma patients, despite their greater average age, demonstrated normal responses to all 3 mitogens and were impaired only in their responses in MLC.

Correlation of in Vitro and in Vivo Assays. When positive and negative responses to the contact allergen DNCB were analyzed versus the patients' responses to the mitogens, there were no significant differences (Table 3). Neither group was significantly depressed to PHA or PWM and both were quite depressed in their responses to Con A. However, there was a very noticeable difference in the responses in MLC. The average response of the nonresponders was less than one-half that of the positive responders. The MLC test was the only in vitro assay that showed a statistically significant difference when the DNCB-positive and negative responders were directly compared ($p < 0.0005$).

Those patients who had a poor skin test response to recall antigens, i.e., a positive response to none or only 1 of the 4...
antigens, showed significantly depressed responses in all 4 assays of in vitro lymphocyte function (Table 3). The groups who responded to skin test antigens were not depressed, or only slightly so, in their responses to PHA, PWM, and in MLC. All groups manifested an impaired response to Con A. It appears that failure to exhibit cutaneous hypersensitivity to common skin test antigens does correlate to poor responses in the in vitro tests of lymphocyte function. However, an increased number of positive skin tests is not necessarily an indicator of better lymphocyte function, but probably just reflects increased prior exposure to these antigens. One-half of the poor responders to the common antigens (7 of 14) were also DNCB non-reactors, while only 7 of 36 patients (19%) who responded to 2 or more recall antigens failed to respond to DNCB.

DISCUSSION

The results presented here indicate that several types of immunological defects in cancer patients can be identified with a coordinated program of in vitro and in vivo testing. The in vivo tests (DNCB response and recall cutaneous hypersensitivity) identified immunological deficiencies in the carcinoma group, although the group’s responses to mitogen stimulation appear to indicate a normal lymphocyte proliferative capacity. They did manifest some impairment in MLC response, similar to the findings of Han and Takita (6). It has been reported that many carcinoma patients have serum factors that inhibit PHA-induced proliferation (11). Suciu-Foca et al. (15) recently reported that MLC and PHA responses in a group of cancer patients of various histological types were significantly lower than in normal controls, and that MLC was markedly inhibited by the patients’ sera. The immunological defect identified by the skin tests in our group may be mediated by a circulating serum factor rather than by an intrinsic defect in lymphocyte function.

The opposite situation appeared with the melanoma patients. Their skin test reactivity was better than that of the carcinoma patients, but their proliferative responses to mitogens were impaired. Catalona et al. (3) reported a series of Stage I melanoma patients who did not display any impairment in PHA responsiveness. However, their melanoma patients with disseminated disease showed some impairment, as did our patients.

These same researchers reported in vitro depression of PHA responsiveness in sarcoma patients, although McMaster et al. (9) could find no impairment in PHA response of lymphocytes from osteosarcoma patients. Our results, while also suggesting that the PHA response in sarcoma patients is not significantly reduced, indicate that the response to Con A is markedly impaired. Thus, it would seem that a battery of in vivo and in vitro analyses may be necessary to determine immunocompetence.

Comparison of skin test results to in vitro parameters of lymphocyte function discloses one very interesting correlation. When compared to DNCB responders, the DNCB non-responders had a markedly reduced response in MLC without any detectable difference in their response to mitogens. Similarly, those patients who demonstrated poor skin test reactivity to common antigens had a marked MLC deficiency as well as an associated diminishment of responsiveness to the mitogens. It appears that failure to respond to DNCB or to common recall antigens in vivo is closely associated with an inability to respond to alloantigens in vitro. It may be that 2 general types of intrinsic lymphocyte functional defects can be postulated, a recognition defect and a proliferation defect. The antigen recognition defect would be represented by those individuals who failed to make an appropriate response to DNCB or in MLC, while demonstrating normal lymphocyte-proliferative capacity in mitogen assays.

A separate proliferation defect appears to be related to the histological type of disease, being most apparent in

**Table 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. tested</th>
<th>PHA Av. cpm</th>
<th>PWM Av. cpm</th>
<th>Con A Av. cpm</th>
<th>MLC Av. cpm</th>
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<tr>
<td></td>
<td></td>
<td>p*</td>
<td>p*</td>
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<td>p*</td>
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<tr>
<td>Control</td>
<td>36</td>
<td>20,773</td>
<td>11,188</td>
<td>12,196</td>
<td>8,097</td>
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<td>DNBCB+</td>
<td>36</td>
<td>17,581</td>
<td>9,044</td>
<td>7,465</td>
<td>7,013</td>
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<tr>
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<td></td>
<td>(85%)</td>
<td>(81%)</td>
<td>(61%)</td>
<td>(87%)</td>
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<td>DNBCB-</td>
<td>14</td>
<td>19,012</td>
<td>8,122</td>
<td>8,244</td>
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<td></td>
<td></td>
<td>(92%)</td>
<td>(73%)</td>
<td>(68%)</td>
<td>(43%)</td>
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<td>Recall skin tests</td>
<td>0 1+</td>
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<td>7,617</td>
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<td>0.005</td>
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<td>7,195</td>
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<td>6,908</td>
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<td>(85%)</td>
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*a Average net cpm of thymidine-3H incorporated by stimulated lymphocytes. Numbers in parentheses, percentage of response to same stimulus when compared to control group. * Level of significance in the Mann-Whitney test when compared to the control group. N.S., not significant at the 0.05 level.

Adjustment for multiple comparisons to a single control will set the 5% level of significance at values of p < 0.01.
patients with metastatic melanoma. Those patients with a diminished cutaneous hypersensitivity to common recall antigens also appear to have a diminished lymphocyte-proliferative capability. It is well known that the secondary immune response is more difficult to suppress than the primary immune response. Therefore, it is not surprising that those patients who displayed a loss of secondary immune responsiveness also demonstrated the most severe in vitro defects in lymphocyte function.

It is rather surprising that Con A detected many proliferative defects not observed in PHA responses, as both mitogens are considered to be specific T-cell activators. Some work has suggested that "T-cell mitogens" may activate different subpopulations of mouse cells (7, 14), and that a subpopulation of human T-cells that is reactive to Con A may be more defective in cancer patients than are the T-cells reactive to PHA. Wybran and Fudenberg (16) suggested that the rosette test distinguishes a subpopulation of T-lymphocytes that is also reduced in patients with disseminated cancers. Whether Con A and the rosette test are examining the same subpopulation has yet to be determined. This study suggests that the mitogen Con A may be more useful for identifying lymphocyte proliferation defects than is the much more widely used mitogen, PHA.

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