Problems Associated With Study of Cell-Mediated Immunity to Human Tumors by Microcytotoxicity Assays

There is a considerable need for assays of cell-mediated immunity to human tumors, which could be used reliably to monitor the course of disease in cancer patients or for immunodiagnosis (i.e., to distinguish patients with cancer of a particular type from patients with other types of cancer, from patients with benign diseases, and from normal individuals). Several studies [e.g., (1-7)] initially suggested that the colony-inhibition or microcytotoxicity assays would be useful for both objectives. All these reports indicated that patients with cancer had significantly more cytotoxic reactivity than did normal individuals against tissue culture cells derived from tumors of the same histologic type (designated histologic type-specific reactivity) and not against cultures derived from normal cells or from tumors of other histologic types. Few or no normal controls were shown to have significant cytotoxic reactivity. Since then, the specificity of the observed reactions and the relative lack of normal reactivity in these assays have been increasingly questioned. In June 1972, in a conference held at the National Cancer Institute (NCI), some investigators presented data indicating frequent cytotoxic reactivity by normal individuals and a lack of complete histologic type-specific reactivity by cancer patients (8-10). Since then, many laboratory workers, including some of those initially obtaining good specificity, have produced similar patterns of results [e.g., (11-14); Fossati G, Canevari S, Della Porta G: Personal communication]. In a recent paper in this Journal, Takasugi et al. (15) described studies with large numbers of cancer patients and controls tested against a wide variety of cultured cell lines, and concluded that the specificity of cell-mediated cytotoxicity assays is not related to the histologic type of cancer.

What are the reasons for the wide range in the patterns of results? Two main possibilities need to be considered. 1) There are critical technical aspects of the microcytotoxicity assays that can significantly affect the results, and different researchers deal with these important technical variables in substantially disparate ways. 2) As stressed by Takasugi et al. (15), the differences seen among laboratory personnel are related to study design, and the problems with the results are due to fundamental aspects of the biology of the human tumor systems. There has been a series of attempts in the last few years to resolve these issues. Two workshops were held, one in 1971 (16) and one in 1974, during which investigators from different laboratories performed their assays with lymphocytes from the same donors and with the same target cells. At the 1972 NCI conference (17) and at a recent conference in London, sponsored by the International Union Against Cancer, representatives from many laboratories discussed at length the differences between the assays and the possible reasons for disparate results. In addition, there have been, and continue to be, some direct comparisons of various tests and technical variables between different laboratories and within the same laboratory. These efforts have identified a series of questions about technical details in the assays, about problems with base-line controls, and about other features in the design of experiments. Several of these questions are now being critically examined in some laboratories. This is an appropriate time to discuss the issues in some detail, and to try to place them in proper perspective.

Technical Problems Affecting Microcytotoxicity Assays

The technical features suggested to be important in the microcytotoxicity assays can be discussed in the context of the test systems themselves, the base line used for calculation of results, the tissue culture cells selected as the target cells and the preparation of the effector cells.

In regard to the assays performed by various researchers, it is important to consider first the objectivity of each test, the ability to quantitate results and particularly the reproducibility of results. The original colony-inhibition and microcytotoxicity assays depend on visual counting of large numbers of cells and are therefore tedious and susceptible to reader bias and other subjective factors. Therefore, many investigators have tried to make their assays more objective by use of electronic counting of target cells (18) or by labeling of target cells with radioisotopes (8, 9, 19). The initial studies were done with only one concentration of effector and target cells, and the results were dealt with qualitatively, as either significantly positive or not [e.g., (2)]. More recently, several workers have stressed quantitative aspects in their tests by using a range of effector to target cell ratios [e.g., (10, 11, 20)]. Such titrations led the Hellströms (20) to conclude that, contrary to their previous reports, patients with advanced melanoma were


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less reactive than patients with more localized or no evident disease.

Regardless of the method used to enumerate target cells remaining at the end of the assay or of the attacker to target cell ratios used, considerable differences among investigators exist because of variations in the calculation and expression of results. The calculation method and statistical manipulations used to determine significant differences can affect the end results, especially in the frequent cases when one is dealing with small numbers of remaining target cells at the end of the assay [see “Discussion” in (17)].

The reproducibility of results from day to day is an important issue when one tries to use these tests to monitor serially patients with cancer. However, scant attention has been paid to this problem. Heppner et al. (12) found considerable variation in the activity of the same normal donors tested repeatedly. We found good reproducibility when assays were run several days in a row with the same normal individual, but wide divergence when run at more widely spaced intervals (8).

One of the main factors making it difficult to assess clearly the reproducibility of the assays is the problem with identifying a suitable and stable base line for calculation of results. The initial studies, and most subsequent ones, used the results obtained with lymphocytes from a normal donor as the base-line control. Once one accepts the possibility for cytotoxic reactivity by some normal individuals (see below), this presents real difficulties in setting as zero the results of one unselected normal donor. Some investigators have tried to avoid this by using the “media control,” i.e., the target cells alone without added lymphocytes as the base line (11, 12, 21, 22). However, this base line does not adequately consider the possibility of nonimmunologic feeder or cytotoxic effects of lymphoid cells on some cells in culture. Significant cytotoxic effects by all lymphocyte preparations relative to the media control in a test against a particular cell line might indicate reactivity by all donors but also might reflect the susceptibility of the target cells to depletion of growth-promoting factors by the added cells. This susceptibility could also vary from day to day, depending on the health of the target cells. We have examined several other types of controls for possible use as standard base lines in the isotopic release assays: unlabeled autologous or other tissue culture cells and X-irradiated lymphocytes. None of these have given sufficiently consistent results. We have therefore decided to use several normal donors in each experiment and to select the results of the least active normal as the base line (Oldham RK, Herberman RB, Djeu JY, et al: Submitted for publication). This controls for nonimmunologic effects of lymphocytes and also allows for the detection of normal reactivity as well as patient reactivity in the test. The presence of lymphocytes in the well reduces the amount of day-to-day variation in control levels with a given target cell. Particularly with the iodo-deoxyuridine assay, there is a considerable amount of fluctuation in media control values between tests. However, even the least active normal base line is not entirely satisfactory, since it is still subject to the variation in the range of cytotoxic reactivity of the few normal donors on a given day. It will probably be best to use a standard source of nonreactive or poorly reactive normal donor cells as the base line. Cryopreserved lymphocytes from the same individual may be suitable for this purpose.

The type of target cells used in the assays is often cited as a major factor influencing the results [see “Discussion” in (17)]. Most studies of the Hellströms, showing histologic type-specific reactivity, were performed with target cells in culture for only short periods (i.e., a few days to a few weeks). It has been suggested that only these short-term cultures are suitable as target cells and that tests with established cell lines show considerably less specificity and more normal reactivity. However, in one study pointing to such differences, only 12 normal donors were tested, and 2 reacted with short-term cultures as well as established cell lines (22). Also, in this work and in two other studies showing no consistent difference between short-term and long-term cultures ([12]; Fossati G, Canevari S, Della Porta G: Personal communication] the media control was used as base line. Therefore, as indicated above, some observations might be due to nonimmunologic, tissue culture effects rather than to the presence or absence of normal reactivity. When we compared the results obtained from short-term cultures with those obtained from established lines, using the least active normal donor as the base line, we found more cytotoxic reactivity by both patients and controls against the established lines, but we also noted a considerable amount of normal reactivity and a lack of histologic type-specific reactivity with the short-term cultured target cells (Oldham RK, Herberman RB, Djeu JY, et al: Submitted for publication). Therefore, it seems rather unlikely that this issue is critical for the disparate results between different laboratories. It is important, however, not to lose sight of the fact that not all tissue culture cells derived from tumors actually consist of homogenous populations of tumor cells. There is a continuing, vexing problem of successful growth of normal cells from the tumors, particularly fibroblasts. Whereas fibroblastic overgrowth can usually be identified morphologically by experienced investigators, the possibility of growth of other normal cells, e.g., normal epithelial cells of a type similar to that of the tumor, is more difficult to exclude. The recent ability to test for tumor development by human cultured cells in nude mice is helpful in this regard. Other methods for identifying tumor cells in culture are also being used with increasing frequency. These include chromosome analysis, search for characteristic markers on tumor cells (e.g., melanin, estrogen receptors, tumor-associated antigens), and release of tumor-associated products into culture medium (e.g., carcinoembryonic antigen, chorionic gonadotropin). However, even this cannot rule out the presence of a mixed cell population in culture. Virtually none of the current immunologic studies are being done with completely characterized, cloned target cells. In addition, some tumor cells in culture, be they short-term or established, may not contain or may lose tumor-associated antigens. This has been observed in various well-studied animal tumor systems.

Another technical aspect of the assays that varies considerably between laboratories is the separation of effector cells from whole blood. Some investigators prepare lymphocytes from heparinized blood by flotation on Ficoll-Hypaque gradients; some use defibrinated blood, sediment the erythrocytes with gelatin, and passage the leukocytes through nylon columns to remove adherent cells; others use other combinations or variations of these procedures. Contaminating granulocytes or monocytes can produce nonspecific cytotoxicity (17). Some workers have emphasized the adverse effects of Fi-
coll-Hypaque sedimentation, indicating that exposure of effector cells to this material may cause artifactual normal reactivity or nonspecific reactivity (e.g., (19, 23)). However, as with the target cell issue this would not appear to be the explanation for the substantial differences between results from various laboratories. For example, the Hellströms switched from another procedure to Ficoll-Hypaque separation and they have not reported less specificity or increased normal reactivity since the change. Other studies, performed with cells separated by gelatin-nylon column, still showed considerable normal reactivity and a lack of histologic type specificity (Fossati G, Canevari S, Della Porta G: Personal communication; Oldham RK, Herberman RB, Djeu JY, et al: Submitted for publication). In addition, at the recent workshop that directly compared methods among different laboratories, the procedure used for lymphocyte separation, although having some effects on results, did not show consistently more normal reactivity or loss of histologic type specificity with cells separated by Ficoll-Hypaque gradients. Despite the failure to demonstrate important differences in results due to separation methods, all routine procedures yield a heterogeneous mixture of mononuclear cells, composed of varying proportions of T, B, and other cells. It is possible that application of the recently developed methods for isolating purified lymphocyte subpopulations will be useful in distinguishing between specific patient reactivity and normal reactivity. The studies reported to date that have utilized such procedures have been somewhat contradictory (22, 24, 25), but many discrepancies may be related to other technical aspects and study design, and also to differences in the separation methodologies. Some cytotoxic reactivity has been associated with non-T cells (23, 26), and it has been suggested that cytotoxicity associated with such cells is actually antibody-dependent, cell-mediated cytotoxicity. However, no clear documentation of this, e.g., by direct demonstration of the antibodies involved or inhibition by materials known to inhibit this mechanism (immune complexes, antiglobulin reagents, aggregated γ-globulin), has been provided.

Specificity of Microcytotoxicity Assays and Occurrence of Normal Reactivity

As summarized above, none of the identifiable technical features can be clearly correlated with the pattern of results obtained in the microcytotoxicity assays. We must therefore consider seriously that normal human cytotoxic reactivity is a real phenomenon and not particularly rare, and also that the reactivity of lymphocytes from cancer patients may not be entirely directed against histologic type-specific, tumor-associated antigens. The study design of experiments in different laboratories should then be carefully examined as a central factor in the controversy. Most reports of microcytotoxicity tests with human tumor systems have been summaries of varying numbers of small, individual experiments. Each experiment typically consisted of effector cells from one normal donor and two or three target cells. The types of target cells were usually derived from the same type of cancer as the patients' normal cells, usually skin or tissue fibroblasts, from the same or different patients; and perhaps cells derived from a different type of cancer. The normal fibroblasts have been the most frequent negative control; few cancer patients had cytotoxic reactivity against these. However, such cells may be quite resistant to cytotoxicity and are not adequate specificity controls. Experiments designed along these lines, although performed many times, would probably not detect normal reactivity and also may not be likely to document clearly the specificity of the reactions. To begin seriously to analyze the specificity of the microcytotoxicity assays, it is desirable to set up experiments involving a large "checkerboard" design, with lymphocytes from several patients with cancer of different histologic types, from patients with benign diseases of the same organs, and from several normal individuals, tested against almost an equally large array of target cells derived from various types of cancer and normal cells. All target cells used in an assay, including those used as specificity controls, need to be susceptible to cytotoxicity by lymphocytes from some patients or normal controls. The use of target cells resistant to cytotoxicity would obviously bias the control results in the desired direction of histologic type specificity. To study directly the frequency and range of normal cytotoxic reactivity, it is particularly necessary to design experiments to look for such reactivity. Several normal donors, not selected because of previous reactivity or lack of reactivity in microcytotoxicity tests, must be tested concurrently, and significant differences between their activities examined. The change in the pattern of results in some laboratories (12, 13); Fossati G, Canevari S, Della Porta G: Personal communication; from apparently good specificity and no normal reactivity to considerable cross-reactivity and normal reactivity, without any changes in test methodologies, can probably be ascribed to changes in study design of the kind we have just described.

Possible Relevance of Data From Experimental Tumor Systems

It may be helpful to look to the experimental animal tumor systems for possible analogies with, and insight into, the complex and difficult-to-analyze human tumor systems. Almost all human cytotoxicity testing involves allogeneic target cells; therefore, the observed immune reactivity would have to be against common rather than individual specific antigens. In the animal tumor systems, common tumor-associated antigens have usually been related to expression of fetal or virus-associated antigens. These common tumor-associated antigens usually have not been restricted to a particular histologic type of tumor, but rather the same antigen might be found in leukemias, sarcomas, and even carcinomas. The best explanation for histologic type-specific tumor antigens might be expression of normal tissue-associated antigens on tumors, but such specificities have not, to our knowledge, been detected in cell-mediated cytotoxicity experiments in animal tumor systems. It should not be too surprising, therefore, to find cytotoxic reactivity against antigens expressed on diverse types of human tumor cells. The possibility of cytotoxic reactivity against individually specific tumor antigens, characteristic of carcinogen-induced animal tumors, needs to be evaluated by tests against autologous tumor cells. However, this is logically a difficult task, which explains why little autologous testing has been done.

Another lesson in regard to specificity of cell-mediated cytotoxic reactions may be drawn from experiments with tumors induced by murine sarcoma virus in mice. This tumor system has been popular in experimental tumor immunology, and many studies of cell-mediated immu-
After the events described thus far, all natural reactivity appears to be directed against virus-associated antigens. Cytotoxicity determined by an inhibition assay, the cytotoxicity detected by a "Cr-release assay was shown not to be related to any of these antigens but rather to those associated with mouse endogenous type-C viruses. This emphasizes the need to define carefully the specificity of cytotoxic reactions, which usually necessitates extensive testing.

Cytotoxicity by lymphocytes from some normal individuals may also be analogous to natural cell-mediated cytotoxicity recently detected in murine tumor systems. Many normal mice and rats have shown specific cytotoxic reactivity against various leukemias, lymphomas, and mammary carcinomas. In the systems described thus far, all natural reactivity appears to be directed against virus-associated antigens. It is intriguing to postulate that some normal human cytotoxic reactivity is directed against antigens associated with ubiquitous viruses. All such natural murine reactivity was initially missed, primarily because it was not sought and because lymphocytes from normal animals were used as the base-line control. Also, the natural cytotoxic reactivity of mice and rats against leukemia cells is mediated by a unique subpopulation of non-T cells, and this provides an opportunity to clearly separate normal reactivity from T-cell-mediated immunity of tumor-bearing individuals.

Conclusions

Microcytotoxicity assays are presently not suitable for diagnosis of human cancer or for reliable monitoring of the course of disease of individual cancer patients. The occurrence of frequent normal reactivity and the consequent difficulties in establishing a constant base line virtually preclude both of these objectives at the present time.

Because of the technical difficulties with these assays, and the findings of normal reactivity and of lack of histologic type specificity, it is often concluded that the reactivity of some individuals is nonspecific (presumably equivalent to nonimmunologic). However, we should not be too hasty in equating failure to detect desired or expected results with failure to detect meaningful, immunologic reactivity. In fact, as pointed out above, from the experience with the animal models, we probably should not have expected to find histologic type-specific, common, tumor-associated antigens. Rather than conclude that results are nonspecific and therefore discard or close our minds to such findings, we should analyze carefully these reactions and try to determine the nature of the specificity. This is not an easy task, but methods are available (e.g., the inhibition assay cited above) to begin such analyses.

Similarly, cytotoxic reactivity by normal individuals should not be ignored but should be directly analyzed. There are some important questions to ask: Is the normal reactivity directed against the same antigens as that of cancer patients? Does the natural reactivity reflect exposure to ubiquitous tumor-associated viruses or reflect an important, natural immune surveillance mechanism against cancer? Or, rather, is the normal reactivity some form of nonimmunologic artifact which might still be eliminated by improved technology? Is the normal reactivity mediated by the same type of cells as the reactivity of cancer patients, and if not, does this provide a basis for practical separation of these activities?

In summary, although the microcytotoxicity assays are not presently providing directly practical and useful clinical information for individual patients, we should not be overly discouraged. We believe that, rather than abandoning these tests, a concerted effort should be made to develop better and more standardized techniques and to analyze carefully the complex series of observations being made. Thus it may be possible ultimately to utilize cytotoxicity assays as valuable clinical measures of cell-mediated immunity to human tumor-associated antigens.

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