METHODS IN CANCER RESEARCH

Edited by

HARRIS BUSCH

VOLUME VIII

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Preface

Among the areas of expanding interest in cancer research, immunology is one of particular current interest. In this volume five chapters are devoted to various aspects of cell-mediated and humoral immunity. Transplantation procedures in tumor immunology are also discussed. Various aspects of membrane immunofluorescence are presented. I am especially appreciative of the helpful advice of Dr. Jan Vaage, formerly at the M. D. Anderson Hospital, Houston, and currently at the Massachusetts General Hospital, Boston, for valuable advice and suggestions in the development of this section.

Some newer areas of interest in virology are detailed particularly relating to satellite viruses, infectious nucleic acids, and subviral constituents. The use of nucleic acids in studies on transformation are also reviewed. It is clear that the presentations in Volumes VII and VIII are part of the evolution of critically useful methods in a number of fields of oncology. Further developments in these fields will be reviewed in future volumes of this treatise.

HARRIS BUSCH

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IMMUNOLOGY

CHAPTER I

COLONY INHIBITION AND MICROCYTOTOXICITY ASSAY METHODS FOR MEASURING CELL-MEDIATED AND ASSOCIATED ANTIBODY IMMUNITY IN VITRO

G. H. HEPPNER

The demonstration of immune responses to antigens associated with tumor cells was achieved initially in animals using methods based on the induction of transplantation resistance (Old and Boyse, 1964; Klein, 1966). The immune responses detected by these methods were thus, by definition, those involved in protection of the host against growth of his tumor. This protection could be passively transferred by immune cells, but not by serum, and so appeared to be the result of lymphocyte-mediated immunity (Klein, 1966). As discussed elsewhere in this volume (see Chapter II), the methods of transplantation have been, and still can be, used to study many aspects of host–tumor interaction. However, these methods are of necessity limited to studies with animals other than humans. Also, the immunity to tumor antigens is complex and involves both cell-mediated and antibody responses,

some toxic, some protective to tumor cells. These interacting responses are difficult to assess *in vivo*. Therefore, *in vitro* methods either have been developed, or borrowed from other areas of cellular immunology, to analyze the immune responses to tumor antigens.

This chapter will discuss a number of assays which are currently being used to detect cell-mediated, and related antibody, responses to tumor antigens. These assays are not easy to perform and require first-hand experience to assess in any detail. This review will also emphasize methods of measuring cell-mediated, and associated antibody reactions, rather than complement-fixing, cytotoxic antibody. However, many of the assays can be used, with modifications, to measure cytotoxic antibodies.

I. Cell Culture Methods in Tumor Immunology

A major motivation in the development of *in vitro* cellular immunity assay methods was the desire to study the immune responses to tumors in terms of functional significance, that is, the ability of immune factors to influence growth and survival of tumor cells. First of all, this requires a method of growing tumor cells in cell culture and second, a way of assessing lymphocyte-mediated tumor cytotoxicity. Rosenau and Moon (1961) were among the first to attempt such techniques. They studied the ability of sensitized lymphocytes from strain Balb/c mice to lyse monolayered cells of strain C3H origin. Comparison was made between the number of target cells (as measured by the number of nuclei released following incubation in 6% citric acid) after a 48-hour incubation with normal versus sensitized lymphocytes. In addition, the ability of sensitized lymphocytes to cause cytopathic effects on the target cells was also noted. Other investigators have also used established monolayers of tumor cells in an effort to detect immune lymphocyte cytotoxicity (Wilson, 1963, 1965; Ulrich and Kieler, 1969). Toxicity has been assessed by release of isotope from damaged target cells (Holm and Perlmann, 1967; Kikuchi *et al.*, 1970), by counting plaques of dead cells (Möller and Möller, 1965; Kikuchi *et al.*, 1970), and by counting the number of surviving target cells after trypsinization (Ulrich and Kieler, 1969). A method of measuring the ability of immune lymphocytes to interfere with the formation of a monolayer of tumor cells has also been described (Nairn *et al.*, 1971). Quantitative growth of cells in monolayer cultures is difficult to reproduce, however, resulting in variable results. The need for a more easily quantitated method of growing tumor cells *in vitro* led to the development of the colony inhibition assays for studying lymphocyte-mediated cytotoxicity.

II. Colony Inhibition Tests

A. The Hellström Colony Inhibition Test

The colony inhibition (CI) test was first developed by I. Hellström (1967). This method, which is shown diagrammatically in Fig. 1, measures the ability of sensitized lymphocytes to reduce the cloning efficiency of tumor cells cultured in 60 × 15 mm plastic petri dishes. The tumor cells are initially cultured

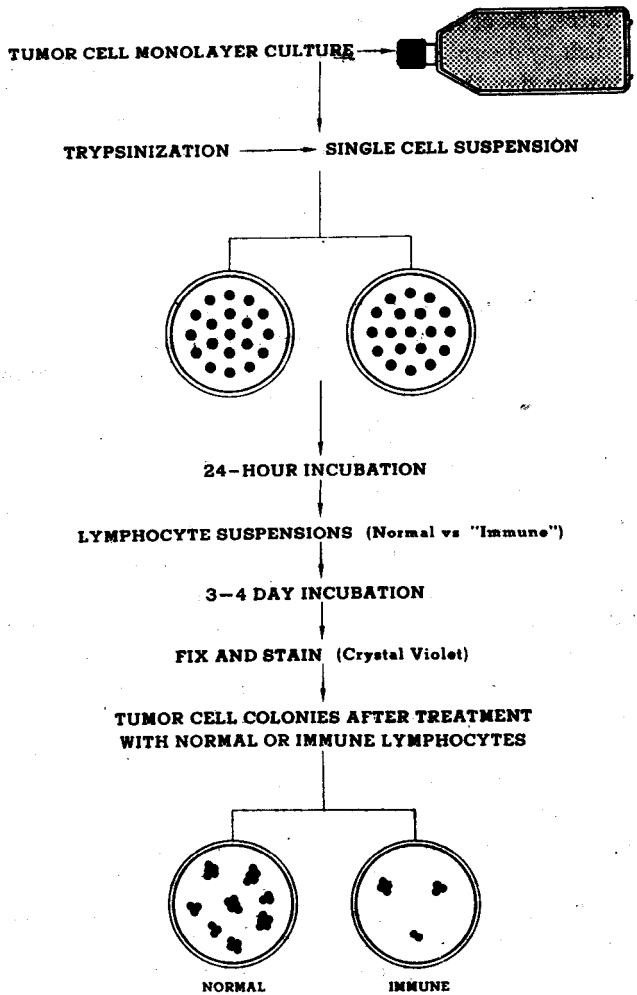

FIG. 1. The Hellström Colony Inhibition Test, (Hellström, 1967.)

in monolayer cultures using a medium appropriate for the particular cell type. When a healthy, growing culture is obtained, but before overgrowth of the cells, the cells are trypsinized off the surface of the culture flask and placed in suspension by careful, repeated pipetting. It is very important that a single cell suspension be obtained. The cells are then pipetted in 4 cc of growth medium into the petri dishes. The number of cells plated per dish depends upon the cloning efficiency of the particular tumor being used. For ease in counting, one would like about 50 colonies per dish to develop in untreated cultures. The cells are then allowed to settle and stick to the surface of the plastic dish by overnight culture at 37°C in a 5% CO_2 in air atmosphere.

The following day the media, and nonsticking cells, are decanted from each petri dish. Suspensions of lymphocytes (see Section IV) are pipetted onto the cells. It is best to carry out a titration of the number of lymphocytes added per dish—having tests with a lymphocyte to target cell ratio of 100:1, 1000:1, and so on. This ratio is calculated on the basis of the number of tumor cells initially plated, rather than the number fixed to the surface of the dish and capable of forming colonies. Indeed, this latter value is not known for certain in any particular experiment until the end. The number of lymphocytes per target cells necessary to achieve significant immune lymphocyte-induced cytotoxicity varies with different systems. In the mammary tumor system a ratio of at least 1000:1 is mandatory (Heppner and Pierce, 1969).

In the CI test the effect of lymphocytes from test animals is always compared to that of cells from control animals. The control lymphocytes may be obtained from normal donors and, better yet, donors sensitized to an unrelated tumor. It is not justifiable to compare cultures incubated with test lymphocytes to untreated cultures. Frequently the number of colonies developing in cultures treated with control lymphocytes will be greater than that in cultures incubated in medium alone. This is believed to be a result of a "feeder" effect of lymphocyte suspensions on the tumor cells (Carrel, 1922). In any event, failure to recognize this may result in a seeming lack of cytotoxicity by immune lymphocytes, which, in weak systems, may only be enough to overcome the feeder effect. On the other hand, lymphocytes from normal human donors may sometimes be cytotoxic for tumor cell cultures for reasons apparently unrelated to sensitization to tumor antigens (see Section IV,B). Since the same effect presumably may also be a factor with lymphocytes from tumor patients, comparison of colony number in cultures incubated with patient lymphocytes to that of untreated cultures may result in false positive readings of cell-mediated immunity.

The lymphocytes are added to the petri dishes in 0.5 cc volumes and incubated for 45 minutes. The medium used for the lymphocyte suspension should not be a growth medium. There is a fine line at this point between allowing for growth of tumor cells but not growth of various cells contained

in the relatively impure suspensions of lymphocytes. Therefore, it is customary to change to a "weaker" medium, say from Waymouth's to Eagle's minimum essential medium (MEM) at this point. We also do not add any serum supplement during the 45-minute incubation period. Certain batches of serum may have nonspecific inhibitors of cell-mediated immunity. Unless one has the facilities, and patience, to pretest all batches of serum for this property, it is best to leave it out at this step. Following the 45-minute incubation period, which is the main time of lymphocyte–target cell contact, medium is added to dilute the culture. Usually 2 cc of MEM is added with no serum supplement, incubated overnight, and then an additional 2 cc containing 30% fetal calf serum is added. With pretested serum, however, one could initially add 4 cc of 15% supplemented medium. Different tumor types may require different amounts of serum.

The tests are now allowed to incubate for 3–4 days. The medium is then decanted, and the sticking cells are washed thoroughly with phosphate-buffered saline (PBS). The cells are fixed and stained in 2% crystal violet in PBS. The stain should be left on the cells from 2 to 5 minutes, depending upon the cell type. Then the dishes are washed thoroughly in distilled water. Care should be taken during the washing and staining procedures. Hard jets of liquid should never be aimed directly onto the cells.

After the plates have dried, the colonies are ready to be counted. This can be done with an inverted microscope. Several considerations enter into scoring colonies. The number of cells making up a colony will differ from tumor type to tumor type. Some tumors will never show large colonies under the culture conditions used in the test. For cells to be considered a colony, that is, descended from a single cell, as opposed to a group of unrelated cells, it is helpful to be able to see some cytoplasmic connection. If this is not possible, the relationship of the cells to each other may be useful. Cell clumps, or groups of cells which were not broken up prior to plating, are generally "tighter" than in colonies and may still look like a piece of monolayer sheet. One other problem in scoring colonies is differentiating between the tumor, target cells, and other cells, such as fibroblasts or macrophages, which may be contaminants of either the tumor cell or lymphocyte suspensions. Plates should be prepared of tumor cells alone and of lymphocytes alone, so that one can see the range of cell types in each suspension. If any cells plate from the lymphocyte suspension, the procedure of preparation should be reviewed (see Section IV). There is no absolute way of distinguishing tumor cells from normal cells in culture. With experience one should be able to eliminate fibroblasts as a consideration. Some tumor cells may have sufficiently different morphology (neuroblastoma), or may produce a particular product (melanoma) so as to ease identification. Otherwise, one tries, for example, in the case of carcinoma, to count only epithelial-appearing