METHODS IN CANCER RESEARCH

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VOLUME II

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Preface

The very warm and interested response to Volume I of this treatise has made it a particular pleasure to present this second volume. Volume I was designed to detail the methods dealing with morphology of tumor cells, transplantation and metastasis, and carcinogenesis. The very important topics of immunology and special products of cancer cells, cell fractionation, and enzymes are presented in this volume. In later volumes, there will be an expansion of the topics of molecular biology, sources of antitumor agents, biology, and therapy, and additional methods for studies on carcinogenesis and molecular biology.

It is apparent from the breadth of these subjects that cancer research utilizes techniques and interpretations which are well beyond the grasp of even the most sophisticated investigator who may be fully informed in one field but necessarily will be limited in other fields. This treatise was designed to make it possible for such highly specialized individuals to clearly grasp other methods used in cancer research and, in addition, it was hoped that less specialized individuals, such as graduate students and postdoctoral fellows, would be able to derive value from both the methods and interpretations presented.

At this point it seems appropriate to express sincere appreciation to the many colleagues who have contributed their time and effort to the preparation of this work; they have striven to keep their presentations within appropriate limits and yet have presented with clarity the important aspects of their subjects. It is also a pleasure to thank the staff of Academic Press for their successful endeavors in providing excellent reproductions of complex figures and most satisfactory bound volumes.

HARRIS BUSCH

Houston, Texas
June, 1967

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CHAPTER I

CANCER IMMUNOLOGY IN MAN

CHESTER M. SOUTHAM

I. Introduction

This chapter considers methods which are or might be applied to the immunological study of human cancer including the study of human cancer tissue in the laboratory and clinical investigation of the cancer patient. It points out some of the technical and biological problems peculiar to human cancer, and how these problems influence the choice of techniques by which human cancer can be studied. It attempts to assess the validity of data that might be or have been obtained by these techniques, but no attempt will be made to review fully the experimental results which have been obtained in this field, since the objective is to consider methodology rather than data. Details of technical procedures will not be described, but references to guide the reader to technical details will be cited.

The subject of cancer immunology includes two separable but interrelated problems, the immunological consequences of cancer, and the effect of immunological reactions on the etiology and pathogenesis of cancer. The techniques which can be applied to the investigation of cancer immunology will be considered in two major categories: those which

involve the study of the patient directly (i.e., clinical investigation), and those which involve the study of the tumor tissues without the direct participation of the patient except as the donor of the tumor tissue.

II. Study of the Patient

Methods for the study of cancer immunology in man differ greatly from those which are applicable to the study of cancer immunology in experimental animals. When patients participate directly in research, the investigator is no longer solely a scientist; he unavoidably acquires a relationship with his patient-subject which is akin to the physician-patient relationship. Although the major objective is the gathering of knowledge of broad applicability, there is often the hope of benefit to the patient himself, and there is always the obligation not to harm the subjects physically or psychologically. These considerations, in addition to purely biological problems, influence the methodology of all clinical investigation.

A. General Immunological Status of Cancer Patients

Since immunological reactions are of many types and mechanisms, there can be no single test which adequately defines immunological status. Rather it is necessary to utilize a series of tests for different aspects of immunological function to obtain what might be termed an "immunological profile" of the individual. Neither the component tests of a complete immunological profile, nor the criteria for evaluation have been defined, but numerous methods are applicable which contribute to such an evaluation. These would include the study of serum antibody reactions, cell-mediated specific immune reactions, and nonspecific cellular and extra-cellular reactions related to host defense.

1. *Serum Antibody Production*

a. Antigens. Antigens used for immunization (the vaccine) in an investigation of antibody-producing capability should be one to which the individual under study, and the population under study, has not had prior exposure (unless the anamnestic response is being investigated). It should provide an effective stimulus by a single injection. The response should be predominantly of the serum-antibody type rather than the cell-associated type. The vaccine should be of reproducible potency and reasonable stability. It should cause little or no local discomfort or systemic illness, and the resulting antibodies should not of

themselves create any adverse reactions. There must be a satisfactory test antigen for subsequent determination of antibody levels, and preferably one which is applicable to various techniques of antibody titration including quantitative methods. The best antigens are therefore ones that are exotic to the subject's usual environment and with which he would not normally be vaccinated. Live viral vaccines have the advantage of propagating within the recipient until they produce a maximal antigenic stimulus, but there are also advantages in the use of a vaccine which provides a precisely known dose of antigen. Examples of antigens that fulfill some of these requirements and have been used in clinical investigation include pneumococcus capsular polysaccharides (Larson and Tomlinson, 1953), hemocyanin—the oxygen-binding protein of invertebrates (Weigle, 1966), heterologous erythrocytes (Rowley, 1950), bacteriophage (Cone and Uhr, 1964; Uhr *et al.*, 1962), and yellow fever vaccine (Wheelock and Sibley, 1965).

b. Serum Specimens. Serum specimens should include at least one collected before the vaccine is given, and serial specimens thereafter at intervals of not longer than every 7 days for the first 3 or 4 weeks to determine the rate at which antibody develops, and thereafter at longer intervals for as long as possible since the disappearance or the persistence of antibody may be of as much interest as its rate of development.

Antibody titrations should be performed simultaneously on all specimens from an individual patient to assure uniformity of reagents and technique. It is highly desirable to include a known positive standard serum in each test run as a check on reproducibility of the technique.

c. Character of Antibody. The character of antibody changes during the evolution of the immunological response. The time curves of the antibody titers of an individual person, will differ considerably with various techniques of antibody titration. For example, following natural infection by Japanese B virus, hemagglutination-inhibiting antibody will appear early in high titer and will persist at low but readily detectable levels for years, while neutralizing antibody will rise more slowly but will persist at high levels for years, and complement-fixing antibody usually appears later and disappears after 2 to 3 months. These differences undoubtedly reflect differences in the chemical and physical characteristics of the antibody molecules, perhaps at sites other than the antigen-binding site. Studies on the physiochemical nature of antibody molecules have only recently been applied to human sera. Simple treatment of the serum with mercaptoethanol before antibody titration, and comparison of the treated and untreated sera gives presumptive data on the relative amounts and rate of formation of 19 S (macro) globulins

and 7 S globulins. The earliest antibody to appear is in large part the mercaptoethanol-sensitive 19 S macroglobulin; after a few weeks the titer is relatively unaffected by mercaptoethanol, as is characteristic of 7 S globulins (Lo Spalluto *et al.*, 1962; Fink *et al.*, 1962).

More precise separation and characterization of molecular species of antibodies can be achieved by techniques such as chromatographic fractionation (Lo Spalluto *et al.*, 1960), preparative ultracentrifugation, and electrophoresis. These preparative methods are so time consuming, however, that comparative time studies in numerous individuals are not practical. The immunoelectrophoresis technique (Crowle, 1961; Grabar and Burtin, 1964) can be used to separate various globulins of an antiserum in a gel matrix, and then to react the preparation against the test antigen to determine the distribution of antibody activity among the various globulin fractions. (This is the reverse of the usual immunoelectrophoretic study of serum proteins, in which the serum after electrophoresis is treated as an antigen and reacted against an antiglobulin serum.)

d. Serologic Techniques. Numerous serologic techniques are available for the detection and quantitation of antibody. They differ in sensitivity, reliability, and technical difficulty. They are discussed in the section on antigenic analysis (see Section III,B), since the choice of technique is usually more critical in studies of antigenic composition than in studies of antibody formation.

When evaluating the ability of a patient to produce antibody, it is often desirable to use more than one technique. There may be differences between individuals that cannot be appreciated by a single technique, since different manifestations of the antigen-antibody reaction are due in part to differences in the character of antibody molecules. For example, in guinea pig antisera, complement-fixing reactions and anaphylactic reactions involve different kinds of 7 S antibody molecules which can be separated on the basis of their electrophoretic mobility (Bloch 1965).

2. *Cell-Mediated Immune Responses*

Specific immunological reactions are not always demonstrated by the presence of circulating antibody. This is true of immune reactions to the tubercule bacillus and most fungi, and many of the allergic hypersensitivity states. These specific immune states are characterized by the development of a delayed response to the intradermal injection of antigen, in contrast to the immediate wheal and erythema reaction which occurs when the immune state is accompanied by circulating antibodies. In the delayed type of reaction, the maximum induration

and erythema at the site of antigen injection occurs at about 48 hours; the immediate type reaches maximum in 10 to 20 minutes and disappears in an hour or two. The delayed-type skin test reaction is mediated by specifically sensitized lymphoid cells rather than by circulating serum antibody. It is not clear whether the cell-mediated delayed hypersensitivity type of reaction is due to a fundamentally different type of immune response or whether the lymphoid cells merely serve as carriers of ordinary antibody molecules. The latter concept has the advantage of simplicity, since it would explain all types of specific immune responses as modifications of a single basic mechanism of antibody production, but there are experimental observations which seem to favor both hypotheses. Regardless of the mechanisms of these responses, the methods of investigation are quite different. Methods for the study of the cell-mediated specific immune responses are less precise, and generally less satisfactory than those available for serological investigation.

The only well-established techniques for the measurement of cell-associated specific immune reactions are *in vivo* studies. They are of two general types—intradermal skin tests and homografts. *In vitro* techniques are being vigorously investigated and will be mentioned in Section II,A,2,c and Section III,C,1, but at present their applicability to clinical investigation is unproved.

a. Delayed Hypersensitivity Skin Tests. Skin tests with microbial antigens such as tuberculin, histoplasmin, coccidioidin, mumps, and other microbial antigens are the only techniques that have been widely applied in clinical studies. Skin reactions to these antigens depend upon prior natural exposure to the infectious organisms, and thus, the investigator has no control or knowledge of the occurrence, the time, or the quantity of antigenic stimulus. Thus, a failure to respond to the skin test may indicate either inadequate exposure to the antigen or a defective immunological responsiveness. Age, socioeconomic status, and geographic origin of the individuals under study greatly influence the probability of exposure, and thus, it may be difficult to obtain a valid control population.

To overcome these major shortcomings of tuberculin-type skin tests as a measure of a person's current ability to produce a delayed hypersensitivity response, it is necessary that the investigator apply the immunizing stimulus as well as the test antigen, just as he does in studies of serum-antibody production. This can be done with certain microbial antigens, if there is reasonable assurance that natural exposure has not occurred. For example, coccidioidin might be a suitable antigenic stimulus for persons living in the northern and eastern United

States, but apparently such studies have not been attempted as yet, and suitable immunizing preparations are not generally available.

Chemical sensitizers can be used in lieu of microbial antigens, and some clinical studies have been done using dinitrofluorobenzene (DNFB). This method has the advantage of permitting the investigator to apply the primary stimulus in known concentrations at a known time, with assurance that few if any of the subjects will have had prior exposure. DNFB presumably serves only as a haptene, becoming antigenic when it reacts with tissue proteins (Eisen *et al.*, 1952). To produce sensitization, a gauze pad, soaked with a suitable dose of the chemical, is applied to the skin and left in place for 24 hours. If a suitable threshold dose is chosen, sensitization can be evaluated on a "yes or no" basis by applying a suitable test dose approximately 2 weeks later. The immune reaction is indicated by the typical delayed-type response. Alternatively, an even smaller dose could be chosen for the primary sensitization, and this same dose could be repeated periodically until the reaction occurred. With this method, immunological competence could be graded in terms of the number of exposures necessary to bring about a response. Both techniques have been used in clinical studies (Uhr, *et al.*, 1960; Levin *et al.*, 1964, Aisenberg, 1966). Other chemicals have been used to study delayed-type skin reactions in experimental animals (Chase, 1954) but apparently have not been utilized for clinical investigation.

b. The Homograft Reaction. This reaction is the other classic example of the cell-mediated type of immune response. Three types of homografts have been used in clinical investigations of immunological responsiveness: skin homografts, lymphocyte transfer tests, and subcutaneous homotransplants of tissue-cultured cancer cell lines. Each presents problems in technique and evaluation of response, and requires a considerable degree of patient cooperation. In spite of these difficulties, each has been used, and each has revealed a depressed immune status in some cancer patients.

Skin homografts (Kelly and Lamb, 1960; Miller *et al.*, 1961) may reveal impairment of cell-mediated immune reactions by a delay in the time required for rejection. It has the advantage over the other two homograft techniques of being the most widely studied, but it has several disadvantages that restrict its usefulness. The procedure requires skill in plastic surgery, and the tolerance of both recipient and donor for an equal period. Reproducibility of the test is poor because there are no generally established criteria for the end point of the test (i.e., graft rejection) and because the same skin donor cannot be used repeatedly.

The lymphocyte transfer test is an intradermal injection of a lympho-

cyte-rich suspension of homologous white blood cells. It was introduced principally for evaluation of the degree of antigenic foreignness between a recipient and the potential donors of kidney homografts. The injection causes an immunological reaction characterized by a small local area of induration and erythema which evolves over a period of about 10 days. This technique has the advantage of simplicity of administration, relatively easy preparation of the inoculum, and repeated availability of the same donors. However, the usefulness of this technique for evaluation of immunological status cannot yet be assessed. There are apparently conflicting reports of the reactions observed in cancer patients (Hattler and Amos, 1965; Aisenberg, 1965; Levin *et al.*, 1966). The interpretation of the reaction is confused because the injected lymphocytes are themselves immunocompetent cells which, at least in theory, are capable of reacting against the tissue antigens of the recipient, and it is not known to what extent the skin reaction is due to graft versus host reaction and what is due to homograft rejection (host versus graft effect).

Homografts of tissue culture cell lines (Southam *et al.*, 1957) offer the advantages of reproducibility of cell mass and genetic composition of the transplant, uniform cell viability, and the availability of cell lines of different growth potentials. The cell cultures are harvested, washed, counted, and the volume of diluent is adjusted to give the desired concentration of cells. When 2 to 4 million cells are injected subcutaneously into a healthy recipient, they produce a nodule which can be measured repeatedly throughout its period of growth and regression. It is easily biopsied by excision, or by needle or small punch which has little effect on the evolution of the growth and rejection process. The disadvantages of cancer cell homotransplants are the necessity for a laboratory to supply the cells, and the psychological effect of the word "cancer." It is possible that in patients who are severely immunosuppressed, as for renal homografts, cancer cell lines could grow progressively and thus cause disease, but in actual experience in over 300 healthy recipients rejection was always prompt, and there was no deleterious effect even when rejection was slow in over 300 cancer patients.

Cancer cell lines are required because diploid cell lines produce no measurable nodule, and even "transformed" cell lines of normal origin rarely produce nodules. Many of the cancer cell lines, however, have a greater growth potential. They achieve limited propagation even in healthy homologous recipients and excite a vigorous inflammatory rejection reaction. The combination of homotransplant growth and host reaction produce the resulting nodule. In healthy recipients, even the most aggressive cell lines such as HEp 3 and RPMI 41 produce a nodule composed predominantly of inflammatory cells by 7 days, and which is