

Formal Discussion of: A Survey of the Tumor Virus Problem from an Epidemiologic Standpoint

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In view of the present acute schizophrenic status of the field of tumor virology, as Dr. Rowe has so aptly put it, perhaps some form of occupational therapy is indicated, such as an all-out effort directed toward the study of a human counterpart of one of the simpler animal neoplastic diseases known to have a viral etiology. One such disease, or rather group of diseases, leukemia, stands out as a prime candidate among human neoplasms for such an undertaking. Many investigators and groups of investigators throughout the world have been working at the laboratory level for several years in attempts to detect viral agents associated with human leukemia, and a number of epidemiologists engaged in studies of human leukemia are now designing their studies so as to bring to light any association with infectious agents or transmissible factors. The interest of both groups stems, of course, from the knowledge now at hand that viruses are etiologic agents of leukemia in both chickens and mice and from the availability of technics and basic concepts developed in these systems which now may be applied to the human problem.

Until recently, little progress had been made in attempts to bring together epidemiologists and laboratory investigators in a concerted effort to determine whether viruses are related to human leukemia. The conference on leukemia in twins, held recently under the sponsorship of Dr. Brian MacMahon, represented an effort to get the ball rolling on such a concerted effort within a limited segment of the leukemia problem. This conference, sponsored by the American Cancer Society, which brings together scientists of various disciplines involved in cancer research in addition to epidemiologists, could provide a major impetus toward launching an all-out effort on leukemia in general with respect to viral etiology, although this would still represent only a limited portion of the over-all tumor virus problem with which this conference is concerned. The time now seems ripe for full exploitation of currently available technologic and conceptual approaches to the study of leukemia.

As Dr. Rowe has emphasized, the animal viral leukemias represent one of the few models in which the virus is tumorigenic in its natural occurrence and in which the classical concepts and approaches to tumor virology still apply. Also, as Dr. Rowe has reminded us, these animal models fall within that limited group of known viral neoplastic diseases for which Koch's postulates still represent a valid set of rules for establishing the relationship between suspect etiologic agents and the disease process.

For example, the etiologic agents of avian and murine

leukemia multiply and are present in tissues and blood, not only during the overt disease but also prior to the onset of the clinical symptoms. In both species, various antigenically distinct viral agents have been found which produce the same clinical picture. The situation therefore may be compared with the poliovirus problem, in which a family of biologically similar but antigenically different agents are to be reckoned with in the consideration of the over-all specific disease problem. The possibility exists that multiple viral agents may be involved in human leukemia, even though all human leukemia may not necessarily be caused by viruses.

Koch's 1st postulate, dealing with detection of the agent during the overt disease, needs some quantitative readjustment, as Dr. Rowe has pointed out, since the agents of most viral diseases cannot be detected in *all* cases. However, with the strong inoculums used in laboratory reproductions of these leukemic diseases, the agents can usually be detected by both electron microscopy and bioassays in test animals of the same species. The fluorescent antibody technic also has proven successful for several avian leukosis viruses and for 2 of the murine agents (Friend and Rauscher strains). Some of these viruses can be cultivated in tissue culture and isolated in pure form, fulfilling Koch's 2nd postulate, although *in vitro* cultivation has not yet been achieved for all of the murine leukemia strains. In the animal systems, the diseases can be reproduced in other animals and the agents reisolated to fulfill Koch's 3rd and 4th postulates.

Unless nonhuman primates or other laboratory animals that approximate the human are found as hosts for testing the last 2 postulates, as in the case of polio, it will not be possible to carry out all of Koch's rules in establishing the etiologic significance of candidate human leukemic agents. However, as emphasized by Koch (see Ref. 2) in his consideration of other human diseases for which suitable test animals were not available, the proof of etiologic relationship can be satisfactorily accomplished within the framework of the first 2 rules.

With regard to the detection of viral agents in human leukemia (the substance of the 1st postulate), the evidence at this time is almost entirely electron microscopic in nature. Dmochowski and associates (7, 8) first reported the presence of virus-like particles resembling the avian and murine leukemia viruses in thin sections of lymph nodes of leukemic patients examined with the electron microscope. With the finding of a marked viremia during the overt disease in avian myeloblastosis by Beard and associates (1), and in murine leukemia by Dalton and

Moloney (4), methods have been worked out by the latter investigators for the examination of ultracentrifuge concentrates of plasma for the presence of virus particles. Using relatively small samples of plasma, 10 ml, and thin-section electron microscopy, Dalton, Moloney, and their associates (6, 11) were able to find well-defined particles having the characteristic ultrastructural morphology of the murine leukemia viruses in about 14% of over 50 plasma specimens from acute childhood leukemia. No such particles were seen in 76 normal specimens, 36 of which were from young children of the same age group. Only 1 control showed "suspicious" particles; these, however, did not meet all of the requirements used for admitting cases to the positive category in the leukemia series.

The introduction of the phosphotungstate negative-staining procedure in the search for viruses during recent years has greatly facilitated the screening of specimens for candidate particles and has made possible the pinpointing of specimens for a more critical examination by thin-section electron microscopy in their final evaluation. Using this method (5) and concentrated fractions from larger volumes of plasma made possible by plasmaphoresis, Dalton, Moloney, and their associates are now finding characteristic particles resembling the murine leukemia viruses in about $\frac{1}{3}$ of the childhood leukemia cases examined (6).

Others, using only the negative-staining procedure for the detection of virus, have reported virus-like particles in most of the leukemic plasmas they have studied [Burger *et al.* (3), Benyesh-Melnick *et al.*¹]. Since different methods were used for concentrating the plasma fractions, and since the findings with the negative-staining technic were not confirmed by ultrastructural studies in thin sections, it is not known whether the latter groups of investigators have developed more efficient methods for concentrating and detecting virus particles, or whether they are including false positives in their tabulations as a result of extraneous material which cannot be distinguished by the less critical, negative-staining, electron microscopic technic. In any event, it is obvious that virus particles resembling the avian and murine leukemia agents are being observed consistently in human leukemic tissue biopsies and blood specimens by several different groups of investigators, and by several different technics. The time therefore appears to be at hand for a concerted effort by epidemiologists and collaborative groups of laboratory investigators, including expert electron microscopists, to establish, epidemiologically, the significance of these candidate virus particles observed by electron microscopy.

A potential immunologically specific tool for the detection of leukemia viruses has been mentioned by Dr. Rowe, namely the soluble complement-fixing antigen found to be associated with avian leukosis virus infection. Whether a similar antigen, detectable by present methods, is associated with murine and human leukemia remains to be determined. Such a simple tool would make feasible

¹ M. Benyesh-Melnick, K. O. Smith, and D. J. Frenbach: Studies on Acute Leukemia. IV. Electron Microscope Findings in Children with Acute Lymphatic Leukemia and in Children with Infectious Mononucleosis. Submitted for publication.

large-scale seroepidemiologic studies on the association of viruses with human leukemia.

As already mentioned, present immunofluorescent methods can be readily applied to several of the avian leukoses and related viruses. In only 1 of the murine leukemias, Friend virus disease, were the standard procedures readily applicable to the identification of viral antigen by specific fluorescent antibody staining. However, Fink and Malmgren (9) have developed modifications of the procedure that make it possible to detect another of the murine leukemia virus family, the Rauscher virus. Although their modified procedure still does not work with the specific antiserum against another murine leukemia virus, the Moloney agent, it has given positive results in some, but not all, cases of human leukemia investigated. No positive results have been obtained to date in more than a score of normal controls. These investigations are still in the preliminary stage.² The antibody used as fluorescent reagent in the preliminary studies was produced in rabbits by inoculating pooled pellet material derived by ultracentrifugation from the plasma of several different leukemic patients. Although it is too early to draw conclusions, the failure of some human leukemic specimens (cells of the bone marrow or buffy coat) to show fluorescence with the pooled serum is consistent with the hypothesis that antigenically different viruses are involved in human leukemia, or that some human leukemias may not have a viral etiology. Another encouraging fact is that one of the strongest fluorescent reactions thus far observed by Drs. Fink and Malmgren involved a case that also showed one of the highest concentrations of virus-like particles when examined by electron microscopy. It seems apparent that the fluorescent antibody technic, in the hands of professional investigators, may be ready soon for large-scale immunoepidemiologic studies involving antiserum prepared from pooled human leukemic plasma specimens. More specific reagents will have to await the successful propagation of one or more isolates of human leukemia virus, if indeed the antigenic material in the human plasmas is of viral origin. None of the candidate human leukemia agents has yet been cultivated for sufficient time, or in sufficient quantity, for definitive study. Lest this sound too discouraging, *in vitro* cultivation has not yet been successfully accomplished for certain of the laboratory strains of mouse leukemia that can be propagated in animals, and it still is not possible to cultivate virus *in vitro* from primary cases of leukemia which appear spontaneously in mice.

Much work remains to be done in satisfying Koch's 2nd rule, but the successful *in vitro* cultivation and purification of human leukemia viruses in practical working quantities could lead to a rapid solution of the human leukemia virus problem, including the proof that such viruses actually exist.

Another tumor virus that is tumorigenic in its natural occurrence and to which Koch's rules apply, namely the mouse mammary tumor virus, has also been singled out by Dr. Rowe. Although this virus was discovered about

² Fink, M. A.: Immunological Studies on Leukemia Viruses. Presented at The Combined Clinical Staff Conference on Viral Etiology of Leukemia, NIH, March 26, 1964. To be published.

25 years before Gross reported the mouse leukemia virus (10), the lack of a practical and efficient method of bioassay has precluded its exploitation as a guide to investigations of similar cancers in humans with respect to viral etiology. However, recent developments in the laboratory of Dr. Kenneth De Ome, which he no doubt will discuss in a subsequent session of this conference, may make a concerted effort on human breast cancer feasible in the not too distant future.

As for the tumor-producing viruses that are not tumorigenic in their natural occurrence, and the multitude of agents that are to be taken into consideration if most viruses actually are capable of initiating neoplasia under appropriate conditions, it will be necessary to devise a new set of rules for relating etiologic agents to the neoplastic reactions before practical epidemiologic studies can be launched. For, as Dr. Rowe has emphasized, when such an agent displays an obligate nonproductive interaction and pathogenesis involves delayed onset of symptomatology, "Koch's model just does not apply."

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