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COMMENTARY

PROSPECTS FOR BIOLOGIC AND PHARMACOLOGIC INHIBITION OF RIBONUCLEIC ACID TUMOR VIRUSES

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Over the past seven decades, many investigators have shown that cell-free agents, ultimately shown to be RNA-containing viruses, induce naturally occurring tumors in chickens, mice, cats [1] and primates [2]. These tumors are generally of tissues derived from the mesenchyme and include the leukemias, lymphomas and sarcomas. In humans, blood leukocytes from some patients with acute myelogenous leukemia contain molecules specifically related to components of oncogenic primate RNA tumor viruses. These molecules include a reverse transcriptase that is biochemically and immunologically very similar to the reverse transcriptases of the woolly monkey sarcoma and gibbon ape leukemia viruses, which are themselves closely related immunologically [2]. Also found in the cytoplasm of these leukocytes are nucleic acid sequences that are related specifically to the genomic RNA of the woolly monkey sarcoma virus, as measured by molecular hybridization [2]. Recently, antigens related to the major structural protein (p30) of the woolly monkey sarcoma virus were found in extracts of these leukocytes [3]. These data suggest that the potential to produce a whole virus related to these oncogenic primate viruses is present in the leukocytes of some patients with acute myelogenous leukemia, and recently we have isolated such a virus [4]. It is therefore appropriate to develop specific biologic and pharmacologic inhibitors of various steps in the life cycle of these RNA tumor viruses. To understand the possible targets for such inhibitors, we need to know the details of viral replication and the mechanisms of neoplastic transformation. We should also understand which of these virus–cell interactions are important in the induction and maintenance of naturally occurring tumors. An important question is whether the continuous production of virus is important to the spread of neoplasia within one individual animal, or between animals. The answers may vary in different species. Unfortunately, many of these areas are poorly understood at present.

Replication of RNA tumor viruses

The life cycle of the RNA tumor viruses begins with adsorption and penetration, followed by synthesis of proviral DNA, integration of this DNA into the host genome, transcription and processing of viral-related RNA, translation of viral proteins, assembly of proteins and RNA, and is completed by envelopment and release (budding) at the plasma membrane. The details of these processes have been the subject of several recent reviews [5–7]. Some of these steps rely on viral-specific components and therefore might be subject to selective inhibition. Other steps apparently rely on host cell machinery, and blockade might be cytotoxic.

In chicken cells, adsorption and penetration depend on a proper match between viral envelope glycoproteins that are subgroup-specific and cell surface receptors [8]. In murine or other mammalian cells, no clear relationship that governs adsorption is known between viral envelope proteins and cell surface receptors. However, neutralizing antisera to murine leukemia virus bind chiefly to the high molecular weight glycoprotein (gp 69/71) of the viral envelope.* The next stage in the life cycle, transcription of proviral DNA from viral genomic RNA, is unique to this class of virus and is therefore a prime target for selective inhibition. With one possible exception [9], we know of no clear evidence that this pathway of information transfer operates in normal cells. In one carefully studied system, viral-specific DNA can be detected in the cytoplasm within 3 hr of adsorption; by 9·5 hr, most of this DNA is present in the nucleus, and by 24 hr it is integrated into the host cell genome [10–12]. Characterization of two temperature-sensitive conditional mutant avian sarcoma viruses has convincingly demonstrated that viral reverse transcriptase is responsible for synthesis of viral DNA [13–15]. After cytoplasmic synthesis, covalently circularized viral DNA is generated; integration can be blocked with ethidium bromide [12, 16]. In the absence of integration, little viral RNA is transcribed, and transformation of cells and production of virus are blocked [12, 16]. These results do not exclude the possibility that other sarcoma viruses might transform cells in the absence of synthesis and integration of complete provirus. A very important further fact has been established by the observation that these two mutants are temperature-sensitive in a function required only in the first 24 hr following infection for subsequent cellular transformation and viral replication [13, 14]. Therefore, in this system at least, reverse transcriptase has no role in the maintainance of the transformed state or in viral production once proviral DNA has been made. Viral RNA is synthesized exclusively by transcription of proviral DNA, since this process is sensitive to low concentrations of actinomycin D [17] and RNA complementary to viral genomic RNA is not found in infected cells, i.e. no RNA → RNA synthetic pathway is known [18]. At

* J. Ihle, personal communication.

low multiplicities of infection, synthesis of viral RNA requires one round of cellular division after infection; thereafter, however, viral RNA synthesis and replication proceed in stationary as well as dividing cells [19, 20]. Viral RNA is synthesized in the nucleus and appears in the cytoplasm within 60 min [21]. No evidence has been presented that any viral-specific transcriptional machinery is involved here, but this possibility has not been completely excluded. Viral RNA is polyadenylated at its 3'-OH end [7]. Since the reverse transcriptase of avian myeloblastosis virus does not transcribe these tracts of poly A *in vitro* [22] and since virus production is blocked by low concentrations of cordycepin [23], an inhibitor of poly A synthesis [24], we suggested that the segments of poly A are added after transcription of viral RNA from proviral DNA [23]. Cellular poly A polymerases that might adenylate viral as well as cellular RNA species have been isolated and characterized [25]. Viral RNA resembles heterogeneous nuclear RNA in overall size and length of the terminal segment of poly A [7]. On the basis of this and other evidence, we have suggested that viral RNA does not undergo the processing that cellular messenger RNA undergoes prior to appearance in the cytoplasm [26]. The steps in this processing are poorly understood. Translation of viral RNA has not been studied adequately to determine whether any non-host machinery may be required. Assembly and release of virions are poorly understood biochemically, but electron microscopic studies indicate that part of the maturation of type-C particles occurs during the process of budding from the plasma membrane [27].

Transformation by RNA tumor viruses

The initiation and maintainance of transformation do not depend on completion of the viral life cycle [28]. Conversely, not all the functions needed for cell transformation are required for viral replication [28]. Studies with temperature-sensitive mutants of avian [28] and murine [29–31] sarcoma viruses and with phenotypic revertants of transformed cells [32–34] indicate that a complex interaction of viral and cellular gene functions is necessary to initiate and maintain transformation. As described above, synthesis and integration of at least a portion of proviral DNA are essential to initiate transformation. In addition, analysis of the physiological characteristics of a series of temperature-sensitive viral mutants has shown that continuous function of several viral genes is necessary for transformation in the avian [28] and murine [29–31] systems. At least one of these functions is expressed within 24 hr of infection [28]. Genetic complementation studies suggest that at least four other functions are required after 24 hr [28, 35]. A clear description of these functions is an outstanding problem in tumor virology and may lead to the development of selective inhibitors of transformation.

Role of the virus in spontaneous animal tumors

The pathways to cellular transformation and viral replication therefore converge in at least one early function (proviral synthesis) but diverge in at least one other early and several late functions. When we consider ways of interfering with naturally occurring oncogenesis in animals, a definition of the role of the virus is necessary in deciding whether and how to inhibit replication and transformation. Are the production and spread of virus important to the initiation and maintainance of neoplasia, or do the tumors result only from the activation of proviral transforming genes? If the spread of infectious virus between animals is important, is it horizontal, vertical (genetic) or congenital spread? Also, is spread of infectious virus within one animal important for the growth of tumors (recruitment) or for the re-initiation of neoplasia after the eradication of tumor cells? Different relationships between virus, host and tumor are found in different species. In chickens, horizontal, vertical and cogenital infection by avian leukosis viruses all occur in domestic flocks, but only congenital infection is of major importance in the genesis of leukemia [8]. In mice, leukemia and mammary tumor viruses are transmitted vertically, and the appearance of leukemia is related to genetic factors that regulate the permissiveness of the host cell to replication of the virus (Fv-1 locus) and possibly the immunologic response of the host to the altered cell surface of the leukemic cell (H-2 locus) [36]. In cats, animal-to-animal transmission of feline leukemia virus produces clusters of disease even in adult animals, and disease may be related to a weak immunologic response to the virus [37–39]. Feline sarcoma virus may also spread horizontally [40]. Although less data are available, the situation in captive colonies of gibbon apes appears to be similar to the epidemiology of feline leukemia [41, 42].* The isolation of a type-C virus from the leukocytes cultured from the blood of a patient with acute myelogenous leukemia [4], and the finding that this virus is immunologically related to the gibbon ape and woolly monkey type-C viruses [4], suggest that horizontal infection may play a role in this form of human leukemia. The lack of epidemiologic evidence for such a role [43] suggests that many other factors may modulate the outcome of infection.

The detection of one mode of transmission does not exclude the possibility that other modes may be important in tumors; for example, mammary tumors can be produced in strains of mice with a low incidence of tumors by foster nursing animals on mothers from strains with a high incidence of tumors [44]. This phenomenon is due to congenital milk-borne spread of virus [44]. Congenital or even vertical transmission could spread disease to the progeny of animals that were themselves infected by horizontal transmission.

The strategy of interference with oncogenesis depends on the virus host relationships. If a potentially oncogenic provirus is passed vertically in the gametes, infection of animals cannot be prevented by blockade prior to proviral integration. Interruption of functions necessary for transformation would be the ideal treatment. However, inhibition of any step required for viral replication might retard or prevent oncogenesis if continuing infection and transformation of normal cells within the organism (recruitment) were vital for the progression of the tumor. Since the presence of naturally occurring virus-neutralizing antibodies correlates with a low incidence of leukemia

* T. G. Kawakami, personal communication.

in mice which inherit the viral genome.* these mice may prevent leukemia by limiting the spread of virus within themselves. In a group of animals passing virus by horizontal or congenital routes, a major objective would be to block viral infection and replication by immunologic and/or pharmacologic means. First, viremic or virus-excreting individuals that serve as a reservoir for infection of susceptibles should be identified, immunized and treated with antiviral agents that act on steps after integration of proviral DNA. Second, these susceptibles should be identified; as an example, individuals who do not circulate neutralizing antibodies and who contact viremic individuals might constitute a group at risk. These susceptibles should be immunized and receive agents designed to interfere with viral entry or synthesis of provial DNA. Third, individuals with tumors should be treated in the same way as the virus shedders, both to eliminate a reservoir of infection and to block possible recruitment. Immunization in this situation means the stimulation of virus-neutralizing antibodies, either by the injection of inactivated virus or of virus envelope glycoprotein (gp 69/71). Of course, tumor-bearing individuals would also benefit from treatment with agents that specifically block transformation functions, or from immunotherapy designed to enhance a cytotoxic response to viral-related neo-antigens on the tumor surface.

Inhibitors of viral functions

Although we can begin to see when specific viral functions should be blocked in various circumstances, unfortunately no selective, potent pharmacologic inhibitors of any of these functions have been found. The dearth of useful compounds may be attributable to the mechanistic similarity of cellular and viral functions, a major problem in the development of antiviral agents in general. At any rate, most of the inhibitors that have been described are toxic to cells or animals; this toxicity complicates the interpretation of observed effects of these compounds in cell culture or in animals. For example, interference with cellular growth impairs cellular transformation and viral replication [19, 20, 45].

Steps leading to both replication and transformation include adsorption, penetration, and synthesis and integration of proviral DNA. Immunization leading to the production of neutralizing antibodies is the most straightforward approach to blockade of adsorption and penetration. Problems of safety associated with the use of attenuated or inactivated oncogenic viruses as vaccines might be overcome by immunization with the high molecular-weight envelope glycoprotein (gp 69/71). Although no pharmacologic inhibitors of adsorption and penetration are known, selective interference with these events is feasible. Fragments, derivatives or analogues of viral envelope glycoproteins might block penetration by competing for binding sites on the cell surface. Different compounds would probably be required for different viral subgroups or types. This situation might be analogues to the specific inhibition of post-adsorption penetration of A-2 influenza virus by amantadine [46].

By contrast, a great variety of inhibitors of reverse transcriptase have been studied; we have recently reviewed this field [47]. Virtually all of the inhibitors of reverse transcriptase described to date also inhibit cellular DNA polymerases. Compounds that act by binding to the enzyme inhibit cellular and viral polymerases approximately equally [47]. As expected, the potent inhibitors are cytotoxic [47]. More selective enzyme-binding agents are badly needed, but it is not obvious where to search for these or what compounds to synthesize. Substrate analogues, template-primer analogues or template-binding agents do not offer the potential for selectivity that enzyme-binding agents do [47]. Guntaka *et al.* [16] have recently found that ethidium bromide blocks the integration of proviral DNA by inhibiting the formation of covalently closed circular duplex intermediates. This compound is not specific; it also strongly interferes with mitochondrial RNA synthesis [48].

Low concentrations of actinomycin D block transformation and replication by inhibiting the transcription of viral RNA from proviral DNA [17]. Of course, this inhibition is nonspecific; cellular RNA synthesis is also blocked. We found that an analogue of adenosine, 3'-deoxyadenosine or cordycepin, blocks the induction of virus from infected but nonproducing cells [23]. These cells contain proviral DNA but do not synthesize whole virus. After treatment with halogenated pyrimidines, the cells produce virus [49]; this phenomenon is called induction and is a model for the study of events in the life cycle that occur after integration of the provirus. Cordycepin blocks induction at concentrations that are not cytotoxic [23]. Inhibition is observed only if the compound is present during the first 24 hr after removal of the inducing agent [23]. We suggested that this compound inhibits induction by blocking the adenylation of viral RNA [23]. At concentrations that are not cytotoxic, cordycepin does not block cellular transformation after infection by murine sarcoma viruses.† This result may simply reflect the fact that a more limited transcription and/or adenylation of viral RNA sequences is necessary for transformation than for viral replication.

No specific inhibitors of translation of viral mRNA are known. We found that interferon inhibits the induction of of virus by halogenated pyrimidines [50], and it is possible that inhibition of translation of viral mRNA is the mechanism of action. However, we* and others [51] found that the intracellular concentration of viral proteins is not lowered by treatment with interferon. These results suggest that interferon may act in this situation by inhibiting the assembly of viral proteins and RNA or the budding and release of mature virus [51]. No other specific inhibitors of viral assembly and release are known.

Several agents of widely varying chemical composition have been reported to inhibit or reverse transformation of cells in tissue culture. The variety of these agents, plus the tendency to inhibit transformation by a variety of tumor viruses and chemicals, suggests that the mechanism of action is on secondary cellular events necessary to initiate and maintain the transformed phenotype, rather than on primary viral-coded functions. Some of these agents include cyclic

* J. Ihle, personal communication.
† A. Wu, unpublished observations.

AMP or its analogues [52], histones or other polycations [53], dimethyl sulfoxide [54], monovalent concanavalin A [55], inhibitors of proteases [56, 57] and hyaluronidase [58]. With the possible exception of a rifamycin derivative [59], we know of no compound that specifically inhibits or reverses transformation by RNA tumor viruses. Pre-treatment of sarcoma viruses with rifamycin derivatives inhibits transformation [45] or leukemogenesis [60] in proportion to the extent of inhibition of reverse transcriptase. Treatment of animals with viral-induced tumors with rifamycins that potently inhibit reverse transcriptase has yielded variable results [61].* One problem is a lack of information on the absorption, distribution, metabolism and excretion of these rifamycin derivatives. Also, as discussed above, the dependence of tumor growth on continuous synthesis of proviral DNA is not clear.

Outlook

Progress toward selective inhibition of the replicating and transforming functions of RNA tumor viruses is likely to be slow, since the details of many of these functions are not understood. Even when a specific viral process is fairly well understood, such as synthesis of proviral DNA, no clues are obvious to guide the search for selective inhibitors. Two approaches may be helpful. One obvious direction is a continuing, combined biochemical and genetic analysis of (a) conditional mutants of transforming viruses and (b) non-transformed revertants of cells initially transformed by RNA tumor viruses. For the analysis of leukemia viruses, a transformation assay *in vitro* would be very helpful; such an assay probably depends on finding and culturing the proper target cell. Another approach is to study how cells restrict the expression of these viral functions. For example, an understanding of how murine cells of genotype Fv-1^{nn} can restrict the replication of B-tropic leukemic viruses [37] might lead us to mimic this regulation. This restriction is genetically dominant [36], suggesting that cells produce a factor that blocks a step that is subsequent to proviral integration. This factor should be characterized. Whenever specific inhibitors do become available, their rational use will depend on a clear understanding of the role of the virus in the initiation and maintenance of the tumor in question.

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