Oncogene (1997) 15, 1877–1888

MEETING REVIEW

Simian virus 40, poliovaccines and human tumors: a review of recent developments

Michele Carbone[1], Paola Rizzo[1] and Harvey I Pass[2]

[1]Cardinal Bernardin Cancer Center, Cancer Immunology Program, Department of Pathology, Loyola University Chicago, Maywood, Illinois 60153; [2]Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201, USA

Recently, wild-type SV40 and/or DNA sequences indistinguishable from SV40 have been detected in specific types of human tumors: ependymoma and choroid plexus tumors, mesothelioma, osteosarcoma and sarcoma. The same tumor types will develop in hamsters after injection with SV40. These findings are interesting in themselves for they could shed light on the pathogenesis of these tumors. These findings also have public health implications. SV40 was found to have contaminated the poliovaccines and the adenovaccines from 1955 until 1963, therefore resulting in the inadvertent injection of millions of people with this tumor virus. Moreover, our society pays a high cost for asbestos causality, a carcinogen associated with the development of mesothelioma. In addition to asbestos, the potential impact of finding another possible cause for mesothelioma (i.e., SV40), as well as the possible pathogenic role of the contaminated poliovaccines, has generated considerable public interest and concern. To discuss these recent findings, the NIH (National Institutes of Health) and the FDA (Food and Drug Administration), organized an International Conference at the NIH, Bethesda, MD, January 27–28, 1997. The association of SV40 with human mesothelioma was also discussed in a special session at the IV International Mesothelioma Conference that was held at the University of Pennsylvania, Philadelphia, PA, May 13–16, 1997. The purpose of this review is to summarize data, from the discovery of the contaminated poliovaccines, to the most recent findings presented at the meetings in Bethesda and Philadelphia, to discuss technical and other problems associated with this research, and the potential for using these findings to develop new diagnostic and therapeutic approaches for SV40-associated malignancies.

Keywords: SV40; poliovaccines; human tumors

Poliovaccines

Until the development of poliovaccines, paralytic poliomyelitis occurred in an endemic fashion for at least as long as there has been written history (reviewed in Melnick, 1996). To appreciate the magnitude of the problem, in 1956, more than 76 000 cases were reported from Europe, the Soviet Union, Canada, the United States, Australia and New Zealand. In 1955 inactivated poliovaccines (Salk vaccines) became available and mass vaccinations began. In 1960 live attenuated poliovaccines (Sabin vaccines) were introduced and by September of 1961, in the USA 60% of people under 60 years of age had received at least one vaccine inoculation (Communicable Disease Center report NO. 248, 1961). Poliovaccines drastically reduced the incidence of poliomyelitis. In the countries mentioned above, in 1967 a total of 1 013 cases were recorded: a drastic reduction. Since 1990 in the US there are only 5 to 10 cases of poliomyelitis per year. These are due to rare reversion of the attenuated vaccine strains to increased neurotropism.

SV40, Poliovaccines and Adenovaccines

Poliovaccines and adenovaccines were prepared in rhesus monkey kidney cells. Bernice Eddy at the NIH investigated the possibility that these cells harbored oncogenic viruses. In 1960 she discovered that the subcutaneous injection of rhesus monkey kidney cells into newborn hamsters lead to the formation of sarcomas at the site of inoculation (Eddy *et al*., 1961).

In 1960, Sweet and Hilleman had also reported the discovery of a new simian virus which grew in cynomolgus and rhesus monkey kidney cells without causing cytopathic effects, but which induced characteristic cytopathic effects in green monkey cells. These authors demonstrated that this virus, which was called SV40, contaminated both the Salk and the Sabin poliovaccines. Individuals injected with the Salk inactivated vaccine developed neutralizing antibodies against SV40, while individuals fed with the oral attenuated Sabin vaccine did not (Sweet and Hilleman, 1960). The presence of live SV40 in the vaccine was explained by the failure to inactivate a part of the contaminating SV40 virions by the formaldehyde treatment used to inactivate the polioviruses. In addition to the poliovaccines, the parenteral adenovirus vaccines used extensively in military and to a limited extent in civilian population in the United States between 1957 and 1960 contained SV40. Furthermore, the adenovirus 3 and 7 vaccines used between 1961 and 1965 contained Ad-SV40 hybrids with segments of SV40 genome which included the SV40 Tag enclosed within their recombinant genome (reviewed in Lewis, 1973).

Soon after the discovery of SV40, Eddy reported that SV40 was the oncogenic virus in rhesus monkey kidney cell extracts responsible for the production of sarcomas in newborn hamsters (Eddy *et al*., 1962), a finding confirmed by several other investigators (Lewis,

Correspondence: M Carbone
Received 28 May 1997; revised 25 June 1997; accepted 25 June 1997

1973). In addition, it was shown that the intracranial injection of SV40 induced ependymomas in hamsters (Kirschstein and Gerber, 1962). Interestingly, *Mastomys*, an African rodent intermediate in size between the rat and the mouse also developed ependymomas when injected subcutaneously (Rabson *et al*., 1962). Although not associated with human diseases to this point, SV40 was found to replicate in humans, because adult volunteers given SV40-contaminated respiratory syncytial virus stocks intranasally developed subclinical SV40 infection (Morris *et al*., 1961). Furthermore, children who received the oral poliovaccines excreted SV40 in their stools for at least 5 weeks following vaccination (Melnick and Stinbaugh, 1962). These findings confirmed that replication of SV40 occurred in humans and raised the possibility that people not vaccinated with SV40-contaminated vaccines could still have become infected by SV40 through the fecal-oral-route. This latter possibility has never been investigated.

It was found that SV40 could infect human cells in tissue culture, and that some of these cells became transformed (Shein and Enders, 1962). Interestingly, SV40 had different replication patterns in different host species. Monkey cells were defined as permissive because they supported SV40 replication, and hamster or other rodent cells were defined nonpermissive, because they did not support viral replication, but they could be transformed. Human cells were unique, because they could support both transformation and low level SV40 replication, i.e. semipermissive state. Additional studies indicated that SV40 DNA replication occurs in hamster cells, but no viral particles are produced (La Bella and Ozer, 1985). In 1964, Jensen *et al*., demonstrated that SV40 transformed human cells were able to produce subcutaneous tumors when injected into human volunteers. These investigators obtained different cell types from terminally ill patients (volunteers), infected and transformed these cells in tissue culture, and re-injected these cells into the original donors (autologous implantation). Other volunteers were injected with various human cell lines which were transformed in culture by SV40 (homologous implantation). These SV40 transformed cells grew as subcutaneous nodules for 2 weeks and then regressed, possibly because of an immune reaction (Jensen *et al*., 1964). These experiments, despite clear ethical problems, indicated that (1) SV40 transformed human cells could grow as tumors when injected into humans, and (2) the immune system was potentially able to control the growth of human tumors induced by SV40.

Epidemiological studies: poliovaccines, SV40 and human cancer

The agencies responsible for the development and safety of these vaccines were confronted with the potential problem of millions of people exposed to a tumor virus by inoculation or ingestion of inadvertently contaminated poliovaccines and adenovaccines.

No illnesses attributable to SV40 were detected during the initial studies of patients who received the contaminated polio or adenovirus vaccines (Lewis, 1973; Shah and Nathanson, 1976). Nevertheless, only a few short term studies have been performed to determine whether recipients of the contaminated poliovaccines, or the children of pregnant women receiving the poliovaccine have developed a higher-than chance number of neoplasms. Furthermore, to the best of our knowledge, no studies have been conducted to determine the possible pathogenic effects of SV40-contaminated adenovaccines.

Only one report describes the long term follow up in patients inoculated with contaminated poliovaccines (Mortimer *et al*., 1981). This study involved 1073 children under 3 days of age with a follow up of 17–19 years (from 1960–62, until 1979). Of these children 920 received the oral poliovaccine and 153 were injected with the inactivated vaccine. Only one of these children (who received the oral attenuated vaccine) developed a malignancy (mixed tumor of the salivary gland) when she was 15 years old, but this patient was not studied for the presence of SV40. Because no excess risk of mortality was observed in these children, and because it became increasingly difficult to continue the follow up (by 1977 5% of the children had been lost to follow up; this number increased to 13% in 1979) the study was terminated. However, the authors cautioned that the 17–19 year follow-up may have not been sufficient to detect an increased risk for developing cancer in these children. Such a time frame, did not approach the 20–40 year latency period usually described for other human carcinogens: for example, mesothelioma from the time of asbestos exposure, or for adult T-cell leukemia for people infected with HTLV-1. Geissler, while in the German Democratic Republic, compared cancer incidence in 885 783 people born between 1959 and 1961 (86% of whom where vaccinated with 'presumably' contaminated poliovaccines), to that of 891 321 people born between 1962 and 1964 a 'majority of whom' received SV40-free vaccines. This study did not detect an overall difference in cancer incidence among the two groups studied (Geissler, 1990). Geissler found an increased incidence of some types of brain tumors (medulloblastoma oligodendroglioma and glioma) and a decreased incidence of others (meningioma astrocytoma and neurinoma) in people 'presumably' vaccinated with contaminated poliovaccines. SV40-like DNA was found by Southern blot hybridization in 14 out of 53 brain tumors of different histologies from the 'presumably' contaminated group, and SV40 large T antigen (Tag) expression in 18 out of 60. A meningioma from a 6 year old girl who was vaccinated after 1961, also had evidence for SV40 Tag expression. As stated in the manuscript (Geissler, 1990), in the first group 'people presumably vaccinated with contaminated vaccines, 1959–1961', many people did not receive contaminated poliovaccines since not all of the poliovaccine lots were contaminated. Among the people who received contaminated poliovaccines, the viral amount was variable because the level of SV40 was different between batches (Fraumeni *et al*., 1963; Shah and Nathanson, 1976; Geissler, 1990). Finally, 14% of people born in those years were not vaccinated at all; however, they were included for analyses because it was not possible to identify them for exclusion (Geissler, 1990). The second group (1962–1964), 'most of whom received SV40-free vaccines', included people who received SV40-contaminated vaccines, since these vaccines were distributed until 1963 (Geissler, 1990; Shah and Nathanson, 1976). In conclusion, Geissler's

study revealed that the administration of SV40-contaminated vaccines did not affect the overall cancer incidence in humans. It is not possible however, to exclude an increase of certain types of rare tumors that could have not been detected in this study. The findings of Geissler that SV40 sequences were present in some human brain tumors confirmed previous reports that SV40 was associated with certain types of human tumors and diseases (Weiner *et al*., 1972; Soriano *et al*., 1974; Weiss *et al*., 1975; Krieg *et al*., 1981; Walsh *et al*., 1982; Stoian *et al*., 1984). However, the limited technology available at that time made it difficult to reproduce the findings and to obtain definitive results. Southern blots with weak bands were published, and the immunofluorescence data were questioned because of a possible cross-reaction with Tags from other DNA tumor viruses (i.e., BK and JC viruses). In those instances in which SV40 virus had been recovered from human specimens (Weiner *et al*., 1972; Soriano *et al*., 1974; Krieg *et al*., 1981) the findings were verbally questioned as possible laboratory contamination, even if no evidence of contamination existed.

At the NIH conference, Strickler, 1997, reported that the incidence of tumors, including ependymomas, and osteosarcomas has not increased since 1973 when the SEER data became available (SEER, Surveillance Epidemiology and End Results, is a program of the National Cancer Institute to collect data on cancer patients on a routine basis. 13.9% of the USA population is comprised in this study). However, Strickler indicated that his study could have not detected small increases in the incidence of rare tumors, such as ependymomas, choroid plexus tumors, and osteosarcomas. In fact, the age-adjusted incidence of ependymomas and bone tumors has shown a 20% increase since 1973 (Susan Fisher, personal communication). In addition, according to Strickler (1997), the enormous increase in the incidence of mesothelioma observed since 1960 (from almost no cases to more than 2,000 cases per year in the US alone), cannot be attributed to contaminated vaccines because these were given mainly to children, and adults had only a minimal risk of having been vaccinated, and if vaccinated, adults would have been more resistant than children to the carcinogenicity of SV40. However, these hypotheses should be reconsidered because (a) An unknown number of adults were given the SV40 contaminated adenovaccines, (b) Adults were vaccinated with SV40 contaminated poliovaccines: 26.3 million people between 20 and 39 years of age, and 8.4 million people 40–59 received these vaccines in the USA alone (Shah and Nathanson, 1976). These were the age groups in which most mesotheliomas developed in the following 40 years; (c) There are no data indicating that adults are more resistant than children to SV40 and adults exposed to asbestos might be at a greater risk.

Reassuring data about cancer risk in people injected with contaminated poliovaccines were presented by Olin, 1997, who reported that in Sweden, in 1957, the SV40 contaminated poliovaccines (manufactured in the USA) were administered almost exclusively to 700 000 children 4–11 years old (In the following years vaccines manufactured in Sweden were used; these vaccines were mostly SV40 free). The incidence of ependymomas and osteosarcomas did not increase in these children until 1993. The exposed children have not yet reached the age (50–70 years old) when the increased risk of mesothelioma is observed, therefore continued surveillance during the next decades is warranted. Linnainmaa *et al*. (1997), reported at the IV International Mesothelioma Conference that SV40 sequences were not present in Finnish mesothelioma, and that Finland who has one of the lowest rates of mesothelioma, did not receive SV40 contaminated vaccines. Similar results were found by S Emri, in Turkey (personal communication). In conclusion, the possibility that SV40 sequences in human tumors originated from the contaminated poliovaccines and the possible pathogenic role of SV40 are presently unclear and are being investigated.

SV40 oncogenesis

The capacity of SV40 to induce tumors is mainly a function of the SV40 large T-antigen (Tag), a 90 kDa protein found predominantly in the nuclei of SV40-infected and/or transformed cells (for reviews see Fanning and Knippers 1992; Cole, 1996). Tag-mediated transformation results from different biochemical activities. Tag promotes transformation by binding and inactivating the products of a number of tumor suppressor genes, which include p53, pRb, p107, p130/Rb2 (for a review see Simmons, 1995), p300 (Avantaggiati *et al*., 1996) and p400 (Lill *et al*., 1997). These tumor suppressor gene products are necessary to prevent the cell from cycling, and in the normal cell they must be inactivated through phosphorylation and dephosphorylation events to permit the resting cell to traverse from G1 to S. By complexing with p53, pRB, p107, p130/RB2 and p300 Tag inactivates these cellular proteins and induces cell division. In addition, by inhibiting p53, Tag inactivates an important check point which in the normal cell arrests mitosis if DNA alterations are detected. If DNA repair does not occur, p53 induces apoptosis and the cell dies (reviewed in Ruley, 1996). Cells which do not have a functional p53 may complete mitosis even in the presence of DNA alterations. This mechanism is considered responsible for the increased resistance to therapy of tumor cells with inactivated p53. Also of great relevance to carcinogenesis is the ability of Tag to cause a great number of structural chromosome aberrations and aneuploidy in every human cell which express Tag (reviewed in Ray, 1995), and such an effect may be related to Tag inactivation of p53. Following infection of human cells in tissue culture with SV40, many cells die, others acquire an extended life span and some of them eventually become immortal (Ray, 1995; Ozer *et al*, 1996). Immortal cells are usually still unable to induce tumors when injected into nude mice, but these cells may become oncogenic if they are transfected with an oncogene (Reddel *et al*., 1989), if they are treated with carcinogens (Rhim *et al*., 1986), or sometimes after many passages in tissue culture perhaps because they accumulate additional DNA alterations (Ray, 1995). It seems possible that, by this latter mechanism, SV40-transformed cells may occasionally accumulate enough chromosome aberrations to become independent from the continuous expression of SV40 Tag for the maintenance of the transformed phenotype. This

hypothesis from several investigators including Geissler, 1990, has never been fully investigated because SV40-transformed hamster cells in tissue culture uniformly express Tag, and if the expression of Tag is silenced the cells revert to the non-transformed phenotype (reviewed in Ozer *et al*., 1996).

Recent experiments, however, suggest that in tumors developing in SV40-transgenic mice, and in human cells in culture, heterogeneous or transient expression of Tag may be sufficient to induce DNA alterations that maintain the transformed phenotype. Ewald *et al*. (1996, demonstrated that transgenic mice expressing Tag under the control of an inducible promoter in the salivary gland developed cellular transformation and hyperplasia by 4 months of age. At that time, hyperplasia regressed when Tag expression was silenced for 3 weeks. When Tag expression was silenced after 7 months of age, the hyperplasia persisted, indicating that Tag had induced some irreversible alteration capable of maintaining the transformed state in the absence of Tag expression. Expression of Tag in only a portion of the tumor cells, as well as a variable intensity of Tag expression in different tumor cells, has also been described in prostate and breast tumors developing in SV40-transgenic mice (Maroulakaou *et al*., 1994). Furthermore, Moorwod *et al*. (1996), demonstrated that human fibroblasts immortalized with SV40 Tag under an inducible promoter remained viable and immortal in the absence of inducing agents, when the very low levels of Tag could not inactivate all of the wild-type cellular p53 and Rb-related proteins. Since no p53 mutations were detected in these cells, these investigators speculated that the interaction of Tag with p53 leads to genomic instability which may be sufficient to promote random chromosomal alterations which maintain the neoplastic phenotype in human cells even at very low levels of Tag expression. Among these genetic alterations, those involving a gene on chromosome 6q designated SEN6 may be of particular relevance to SV40-mediated transformation of human cells (Banga *et al*., 1997). Of interest, alterations of chromosome 6q are frequent in human mesothelioma (Bell *et al*., 1997), a malignancy associated with SV40 infection (Carbone *et al*., 1994).

In conclusion, extensive experiments in hamsters indicate that the continuous expression of the SV40 Tag is required for the induction and the maintenance of the transformed phenotype. However, recent experiments in SV40 transgenic mice and in human cells in tissue culture suggest that in these systems the continuous expression of Tag may not be a stringent requirement for the maintenance of the transformed phenotype.

SV40 and mesothelioma

The SV40 small t antigen is a 19 kDa protein found predominantly in the cytoplasm of infected and transformed cells. Small t shares 82 aminoacids at its aminoterminus with Tag; the remaining 92 amino acids are unique. Small t enhances the transforming capacity of SV40 by increasing the production of Tag (Bikel *et al*., 1987); by contributing to the complete inactivation of cellular p53 (Tieman *et al*., 1995); and by stimulating mitosis in quiescent cells (Cicala *et al*., 1994), an effect that may be mediated by the ability of small t to induce AP-1 (Frost *et al* 1994). In 1979, Lewis and Martin demonstrated that small t mutants injected subcutaneously (sc) into hamsters induced sarcomas with a prolonged latency compared to wild-type (wt) SV40 (Lewis and Martin, 1979). Later, Dixon *et al*. (1982) discovered that about 15% of the hamsters injected sc with SV40 small t mutants developed tumors in the abdominal cavity, which at that time were interpreted as metastases from the sc sarcomas. These abdominal tumors never developed following injection of wild-type (wt) SV40 (Dixon *et al*., 1982). Further investigations of these abdominal tumors induced by small t mutants, revealed that these were not metastases, but true histiocytic lymphomas originating from a specific subpopulation of mononuclear phagocytes (Carbone *et al*., 1989). We were intrigued by these findings because it appeared that small t mutants might have a particular tropism for these cells. We tested this hypothesis by injecting hamsters intracardially with small-t mutants to expose most different cell types. All of the hamsters injected developed abdominal lymphomas of histiocytic or of B-cell origin, indicating that SV40 small t mutants preferentially transform these cell types (Cicala *et al*., 1992).

Concurrently with the small t mutant experiments, a control group of hamsters was injected with wt SV40. We were indeed surprised when 60% of these animals developed pleural mesotheliomas (Carbone *et al*., 1991) because viruses had never been previously associated with mesothelioma in mammals (Harold L Stewart, Registry for Experimental Cancer, National Cancer Institute, USA, personal communication). When we injected SV40 directly into the pleural space, 100% of the animals developed mesothelioma in 3 to 6 months (Cicala *et al*., 1993). Mesotheliomas did not develop in hamsters injected with SV40 through the femoral vein (these animals developed sarcomas, lymphomas and osteosarcomas; Diamandopoulous 1972). This suggested to us that SV40 reached the pleura and/or the pericardium through the external surface of the needle used during intracardial injection, and that only a little amount of wt SV40 may be needed to induce mesothelioma, compared to the amount required to induce other tumor types. Small t plays an important role in the induction of mesotheliomas, since these tumors did not develop following the intracardial injection of SV40 small t mutants (Cicala *et al., 1993)*.

Mesotheliomas are tumors of the serosal cells of the pleura, the pericardium, and of the peritoneum (for a review see Pass *et al*., 1996). In humans, these tumors were almost unknown until the second half of this century. Since then, the incidence of mesothelioma has increased enormously, to more than 2000 cases per year in the US (Price, 1997), more than a thousand cases in the UK (Peto *et al*., 1995), and 800 cases per year in Italy (Luciano Mutti, personal communication). This increase is real, and not the result of incorrect diagnosis before 1950, because no evidence of background mesothelioma was found in a review of the records of all lung cancers treated at the Massachusetts General Hospital in Boston from 1896 until 1991 (Mark and Yokoi, 1991). The continued rise in incidence in mesothelioma has been related to the widespread use of asbestos during the last 50 years. Although the association between asbestos and

mesothelioma is indisputable, at least 20% of mesothelioma occur in people with no history of asbestos exposure (Roggli *et al*., 1992). Furthermore, among people heavily exposed to asbestos, fewer than 10% develop mesothelioma (Roggli *et al*., 1992), suggesting that other unknown factors may render certain individuals more susceptible to the carcinogenicity of asbestos.

Could SV40, by acting independently and/or with asbestos, be related to the enormous increase of mesothelioma in the second half of this century? There are some data that lend support to this hypothesis: (1) the increase in mesothelioma incidence occurred after millions of people, including adults, were injected with SV40-contaminated vaccines; (2) tissue culture experiments indicated that asbestos facilitated transformation of mouse cells by plasmid DNA and by SV40 (Appel *et al*., 1988; Dubes, 1993); and (3) mesotheliomeas developed in hamsters injected with SV40 (Cicala *et al*., 1993).

SV40 and human ependymoma and choroid plexus tumors

While we were investigating the possibility that SV40 was associated with human mesothelioma, Bergsagel *et al*. (1992) described the first clear evidence that SV40 was associated with human ependymomas and choroid plexus tumors (choroid plexus cells are a specialized type of ependymal cells). By using the new technique of the polymerase chain reaction (PCR), they detected SV40-like sequences, and SV40 Tag immunostaining in most of the ependymomas and choroid plexus tumors they studied. Samples from other types of tumors were negative suggesting that the association of SV40-like and ependymoma/choroid plexus tumors was specific. Because the entire viral genome was not sequenced, the term SV40-like was used to entertain the possibility that these sequences were related to an as yet unknown virus closely related to SV40. The findings were extended by Lednicky *et al*., (1995a) who amplified sequences corresponding to SV40 Tag, the VP1 capsid protein, and the viral regulatory region in human choroid plexus tumors. Furthermore, they succeeded in isolating SV40 from one choroid plexus tumor (Lednicky *et al*., 1995a). Sequence analyses of the rescued virus revealed a regulatory region containing only one 72 bp repeat, and nucleotide changes in the C terminus of Tag. These characteristics clearly distinguished this virus from laboratory strains of SV40. The presence of only one 72 bp repeat in the regulatory region appears common in SV40 isolates from monkeys before passage in tissue culture (Ilyinskii *et al*., 1992), and passage in culture favors the selection of mutants with a duplication of the 72 bp repeat because these viruses replicate more efficiently (Lednicky *et al*., 1995b). Thus, all of the known laboratory strains of SV40 contain two 72 bp repeats in the enhancer region. The occurrence of two 72 bp repeats in monkeys may not be of particular advantage because monkeys are able to prevent SV40 replication through their immune system (von der Weth and Deppert, 1992). However, monkeys are also infected with SV40 strains with repeated enhancer motifs, some containing two 72 bp repeats (John Lednicky, personal communication).

The finding that some SV40 viruses contain only one 72 bp repeat and that these viruses replicate less efficiently in monkey cells in culture is of potential relevance for vaccine preparation. Some poliovaccines are still prepared in primary monkey kidney cells. These cells, however, are tested for SV40 by propagating the cell cultures for several weeks and testing for morphologic evidence of SV40 infection (i.e., vacuolization and cell lysis). It cannot be completely excluded, however, that SV40 virions with only one 72 bp repeat, if present at a low copy number, may escape detection by this method, because it could take a very long time before they induce the morphologic changes which are characteristically induced by 'wild-type SV40' (i.e., SV40 with two 72 bp repeats). It may be advisable, therefore, to consider testing these vaccines also with the more modern and sensitive PCR to exclude the presence of SV40 virions which replicate less efficiently, or to use established monkey cell lines (such as Vero cells) or human cells, to prepare vaccines. More recently the presence of SV40-like sequences in ependymomas and choroid plexus tumors has been confirmed by Martini *et al*. (1996). Martini *et al*. also found SV40-like sequences in other types of brain tumors, confirming previous findings (Weiss *et al*., 1975; Krieg *et al*., 1981; Walsh *et al*., 1982; Stoian *et al*., 1984; Geissler *et al*., 1990), and in sperm. In contrast to the findings of Martini *et al*. brain tumors – other than ependymoma and choroid plexus tumors which were not studied – tested negative for SV40 in a different laboratory – (de Villers 1997), and SV40-like sequences were not detected in sperm from HIV-positive patients in another laboratory (Griffiths and Weiss, 1997). These discrepancies are presently unclear, and may reflect geographic or technical differences.

SV40 and human mesothelioma

Our finding that SV40 preferentially induces mesothelioma in hamsters (Carbone *et al*., 1991; Cicala *et al*., 1993), and the finding of SV40 sequences in the same types of human brain tumors that develop in hamsters following SV40 injection (Bergsagel *et al*., 1992), led to PCR analyses of human mesotheliomas. We used the same PCR approach which was used by Bergsagel *et al*. (1992). The primers amplify the Rb-pocket binding domain of Tag (Figure 1), which is the region that binds pRb, p107, and p130/Rb2. Because the association of Tag with these cellular tumor suppressor genes is thought to be important in SV40-mediated transformation, this region of Tag would be unlikely to be deleted and/or mutated if Tag played any role in the development of these tumors. We studied 48 mesothelioma frozen samples collected and frozen in the operating room at the NCI by one of us (HI Pass), along with 30 non-mesothelioma samples. We took several precautions to prevent PCR contamination, including extracting the DNAs and assembling the PCR reaction in one building, and running the PCR and the Southern blot experiments in a separate building. Two investigators were involved in these experiments and equipment and/or reagents were not exchanged among the two laboratories. When we found SV40-like sequences in 29 of 48 mesotheliomas and in one of 30 non-mesothelioma tissues, we wanted to be sure that we

had not picked-up sequences of other DNA tumor viruses (i.e., BK or JC viruses) which had been found to be associated with human tissues (reviewed in Monini *et al*., 1995). Sequence analyses of five mesotheliomas – performed by a third investigator in a different laboratory which did not work with viruses and/or plasmids containing Tag – indicated that the amplified sequences corresponded to SV40 (Figure 1). Tag expression was detected by immunohistochemistry and Western blot using the anti-Tag pAb419 which is specific for SV40 Tag and does not recognize Tags from BK or JC (Marshall *et al*., 1991), and patients sera contained antibodies to Tag (Carbone *et al*., 1994). The finding of Tag-protein expression in some human mesotheliomas further decreased the possibility of PCR contamination. Several other laboratories have replicated our results in mesothelioma (Cristaudo *et al*., 1995; Pepper *et al*., 1996; De Luca *et al*., In press, Griffith and Weiss, 1997; Galateau-Salle, 1997; Mutti L, personal communication; Testa JR., personal communication). None of these laboratories had previously worked with SV40. Recently, we (Carbone *et al*., In press) and others (De Luca *et al*., in press) have shown co-immunoprecipitation of SV40-Tag with cellular p53, pRb, p130/Rb2, and p107, indicating that Tag is biologically active in these tumors, and that it may contribute to carcinogenesis.

One paper has reported negative findings for SV40-like sequences in human mesothelioma (Strickler *et al*., 1996). These findings were obtained from 50 archival formalin fixed and paraffin embedded samples. It has been suggested that the different technical approach used in K Shah's laboratory may have resulted in false negatives (Pepper *et al*., 1996). It is also our experience, and that of other investigators (Lednicky *et al*., in press), that it is difficult to study SV40 in archival formalin fixed human tumors, and it is preferable to use frozen tissue. It is also possible that these mesothelioma samples were really negative. We recently reported a significant variability in the incidence of SV40-like sequences in osteosarcomas from different regions (Carbone *et al*., 1996) and it seems possible that the same might be true for mesotheliomas. These two possibilities, false negatives versus true negatives, could easily be addressed by testing these same mesothelioma samples in a different laboratory, or by testing the frozen specimens if available.

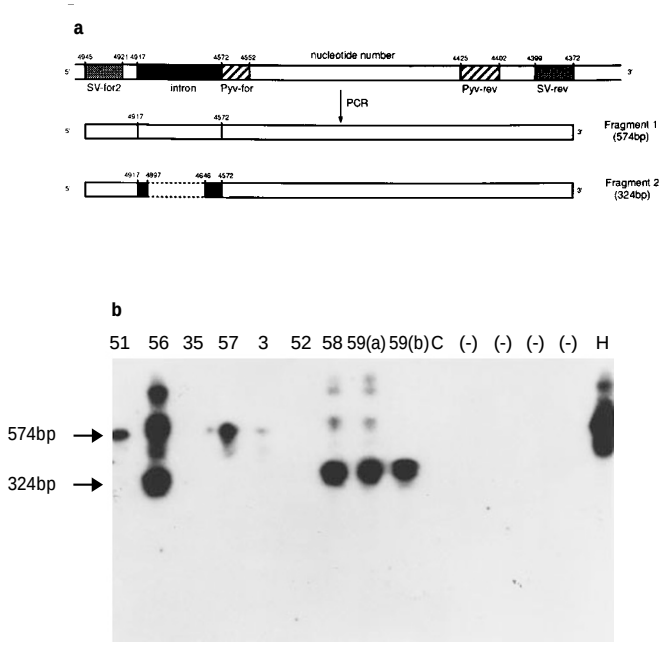

Figure 1 (**a**) Region of SV40 Tag amplified to detect SV40-like sequences in human tumors; some of the primers used are indicated. Some primers are specific for the RB-pocket binding domain of Tag (PYV.for and PYV.rev), others amplify also the intron region of Tag (SV2.for and SV.rev). (**b**) Southern blotting hybridization of the PCR products obtained using the primers SV2.for and Sv.rev in human osteosarcomas. In addition to the expected 574 bp product, some human osteosarcomas contain shorter sequences which are amplified with these SV40-specific primers and which hybridize with an SV40-probe. Sequence analyses indicated that these shorter sequences correspond to Tags with deletions within the intron region. The location of the deletion is indicated in **a**. For a detailed description of these primers and of the PCR conditions, see Carbone *et al*. (1996). This figure was modified from Carbone *et al*. (1996)

SV40 and bone tumors

When we published our paper describing SV40-like sequences in human mesotheliomas, we were intrigued by the coincidence that mesothelioma and ependymoma were also specifically induced by SV40 in hamsters. The other tumor types induced by SV40 in hamsters are lymphomas (mostly true histiocytic and B-cell lymphomas), osteosarcomas, and sarcomas (the latter however develop only following sc injection at the site of inoculation where a very high concentration of SV40 was present).

We analysed a total of 345 different human specimens, of which 145 were bone tumors. The specimens were tested in four different laboratories: Garcea's laboratory at the University of Colorado in Denver CO, my laboratory which at that time was at the University of Chicago IL, Pass's laboratory then at the NCI in Bethesda MD, and Procopio's laboratory at the University of Chieti in Italy. All of the non-bone tumor samples but one (a specimen from a patient with neurofibromatosis type 1) tested negative for SV40 (Carbone *et al*., 1996). One third of the human bone tumors tested positive for SV40. These bone specimens were blindly tested in the four labs, and only after all the labs completed the experiments was the code identifying the specimens broken and the results compared. The results were reproducible in the four laboratories, with the exception of 10 samples which tested positive in some laboratories and negative in others, – all in the bone tumor group – these specimens were classified negative (most likely these were false negative due to some technical problem). For 24 specimens in the bone tumor group – 14 positive for SV40 and 10 negative – there was sufficient material for a second DNA extraction. The results from the second DNA extraction – performed in a different laboratory – matched those obtained previously (Carbone *et al*., 1996). The presence of SV40 in human bone tumors has been recently independently confirmed by others (Lednicky *et al*., in press).

Technical concerns and working hypothesis to determine the origin of SV40 in human samples

The technical concerns associated with these experiments include: (a) that reports of SV40 in human

tumors may result from PCR contamination; (b) that the sequence identified by PCR may belong to a different virus than SV40; (c) that a very sensitive method, such as PCR, is required to detect SV40 DNA in human tumors.

(a) Contamination could originate from the SV40 DNA itself, from many plasmids containing various portions of the SV40 genome, or more likely occur following a few successful and clean PCR experiments because of contamination from one of the amplified reactions. It cannot be excluded that some positive results might be ascribed to such contaminants. However, it is very unlikely that all of the positive results for SV40-like in some specific types of human tumors are the result of PCR contamination because: (1) Many laboratories, most of which had never worked with SV40, reproduced these findings in various parts of the world (Bergsagel *et al*., 1992; Carbone *et al*., 1994, 1996; Cristaudo *et al*., 1995; Lednicky *et al*., 1995a; Pepper *et al*., 1996; Griffiths and Weiss, 1997; Galateau-Sallè, 1997; De Luea *et al*. in press; Pass, 1997; Lednicky *et al*., in press; Mutti L, personal communication; Testa JR, personal communication). (2) Wild-type SV40 was rescued from one human tumor, and sequence analyses demonstrated that it was different from any known laboratory strain of SV40 (Lednicky *et al*., 1995a). (3) Positive and negative results were reproducible in four different laboratories in blinded experiments (Carbone *et al*., 1996). (4) Deletions and mutations in the SV40 sequence were found in some human tumors, in addition to tumors showing the wild-type sequence (Figure 1) (Carbone *et al*., 1996; Lednicky *et al*., 1995a; Lednicky *et al*., in press). This is not characteristic of contamination, when the same band/s is/are always sequenced. (5) Tag mRNA and protein expression were detected in some human tumors (Bergsagel *et al*., 1992; Carbone *et al*., 1994; Martini *et al*., 1996). These results should put to rest the issue of contamination.

It should be underscored that all precautions must be taken to prevent the possibility of PCR contamination, and that when these precautions are taken this risk can be significantly reduced and if contamination occurs readily detected. For this reason, the PCR is used in many research laboratories, and patients are treated because of PCR-based diagnoses.

(b) Let us now consider what these SV40-like sequences may represent. In some cases the sequences are from SV40, because the virus itself could be rescued (Lednicky *et al*., 1995a), or because extensive sequence analyses demonstrated that it was SV40 (Lednicky *et al*., 1995a; Carbone *et al*., 1996; Pass, 1997, Lednicky *et al*., in press). But for the majority of cases, in which extensive sequence analyses were not performed, we still prefer the term of SV40-like to include the possibilities of a human virus related to SV40, or a recombinant SV40 virus, or even a cellular homologue of Tag. Recent experiments in Dr Pass's laboratory revealed that different sets of primers for the SV40 genome resulted in a different percent of positive results when testing human mesotheliomas (Pass, 1997). The SV3.for and SV.rev primers amplified SV40 DNA in 90% of the samples; the PYV.for and PYV.rev primers amplified SV40 in 70% of the DNAs; the SV2.for - SV.rev primers amplified SV40 in 25% of DNAs; the 7/8 primers for the carboxyterminus of Tag amplified SV40 in 38% of the DNAs; the RA1 and RA2 primers for the regulatory region of SV40 amplified SV40 in 50% of the samples (for the sequence of these primers see, Bergsagel *et al*., 1992; Lednicky *et al*., 1995a; see also Figure 1). Overall, 24% of 42 patients showed amplification with all sets of primers. In these experiments, the identity of the PCR product was confirmed by restriction enzyme digestion, Southern blot hybridization, and DNA sequencing (Pass, 1997). Interestingly, very similar results were reported by Robin Weiss at the NIH conference of January 1997: 100% of the 18 samples he studied tested positive for SV3.for and SV.rev primers, and 20% were also positive for the SV2.for SV.rev primers (Griffith and Weiss, 1997). It is presently unclear why different sets of primers result in a different percent of positive samples. Mutations and deletions of the SV40-like genome may occur in human tumors (Carbone *et al*., 1996). Mutations are unlikely to occur in the Rb-pocket binding domain of Tag because of the relevance of this region in Tag-mediated cell transformation. This hypothesis is supported by our findings in osteosarcomas, in which deletions were detected in the intron of Tag, and never in the Rb-pocket binding domain (Figure 1, and Carbone *et al*., 1996). Accordingly, the set of primers specific for the Rb-pocket resulted in the higher percent of positive results in mesotheliomas (Griffith and Weiss, 1997; Pass, 1997). However, the percent of positive results when using some sets of primers for the Rb-pocket binding domain (i.e., SV3.for and SV.rev, and PYV.for and PYV.rev, see also Figure 1), seems too high to us. These primers can amplify Tag sequences from other papovaviruses, and even Southern blot hybridization with specific probes may not be sufficient to distinguish the SV40 Rb pocket binding domain from that of other papovaviruses Tags. A likely candidate could be the Tag of BK virus, because this virus has been reported to be ubiquitous in humans and to be present in many human tumors, especially brain tumors (reviewed in Monini *et al*., 1995). It should be noted, however, that these findings were not reproduced in the laboratory of K Shah who reported zero incidence for BK in human brain tumors (Ray *et al*., 1994). The SV40 and BK Tags are very similar, but a 9 bp insert present in the Tag of BK allows the distinction between the two. For this reason we recommend always to sequence the PCR products when using the PYV.for - PYV.rev and the SV3.for - SV.rev sets of primers. It is also possible that the sequence detected by these sets of primers belong to a recombinant BK-SV40 virus, or a recombinant between SV40 and another unknown virus. Such a recombinant would maintain the RB-pocket binding domain of Tag because of its capacity to transform human cells. There is no evidence to suggest that such a recombinant virus exist. However, recent data indicate that human specimens which contain SV40 are also co-infected with BK (Martini *et al*., 1996). Another possibility is that SV40 is not just a monkey virus. SV40 might be endemic in the human population and be occasionally present in human tissues in such a low copy number to require PCR amplification for detection. It seems possible that such an endemic virus could accumulate various mutations and deletions, except – for unclear reasons – than in the RB-pocket binding domain of Tag. However, there are no data to

support this hypothesis. In conclusion, the hypothesis which has yet to be ruled out is that the sources for SV40 and SV40-like sequences in humans are the contaminated polio and adeno vaccines. Other possibilities cannot be ruled out.

It should be noted that the presence of SV40 in tissues from patients without tumors, or in normal tissues from cancer patients has not been carefully investigated. A few normal tissues have been studied with the PCR in various laboratories including ours, and most of them tested negative for SV40. But the limited resources available have limited these studies. It is also difficult to interpret these results because SV40 may be present only in specific cell types, therefore, a negative result from a given tissue does not exclude that other cell types in the same individual may contain SV40.

It is very unlikely that every person that contains SV40 in some of their cells will develop cancer. Probably, most people with SV40 or SV40-like sequences in some of their cells will not develop cancer, and cancer may be a rare complication in those who are exposed to additional carcinogens, such as asbestos. If our hypothesis is correct, there should be a large pool of individuals that contains SV40 or SV40-like sequences in some of their normal cells.

(c) We finally want to address the issue of sensitivity, and specifically why the very sensitive technique of the polymerase chain reaction is required to detect SV40 DNA in human tumors. Bone and brain samples are usually tiny biopsies taken and used mostly for diagnosis. Therefore, the minimal amount of residual tissue which is available for research can only be analysed by PCR. Mesothelioma samples, on the other hand, are occasionally larger. Figure 2 shows the immunostaining for Tag in human mesothelioma. It can be easily appreciated that the percent of positive cells varies considerably in different tumors. This may indicate that not all of the cells present in the section are malignant, or that not all of the malignant cells express Tag. Both hypotheses may be correct. Figure 3 shows a cytokeratin stain for mesothelioma. Cytokeratin stains mesothelial and epithelial cells, but the reactive normal stromal cells are not stained. It is easy to appreciate that the vast majority of cells in a mesothelioma specimen are normal stromal cells (cytokeratin negative) infiltrated by various amounts of malignant mesothelial cells (cytokeratin positive). It must be underscored that the number of malignant cells present in a section is not related to the degree of malignancy, and survival among these patients is comparable. It should also be noted that Figure 3a and d are from the same patient, actually from the same slide. These are just two different regions of the slide, one with a preponderance of tumor cells and one with almost no tumor cells. We divided the slide in two halves and extracted the DNAs and tested for SV40-like sequences. The half with many tumor cells tested positive, the other half tested negative. If the two halves were sent to two different laboratories, such results would have been interpreted as conflicting data, when in fact both laboratories would have been correct. Figures 2 and 3 also suggest that we may need PCR to detect SV40 in mesotheliomas, because often there are not enough malignant cells in a given sample to be detected by a less sensitive method. However, in those samples in which there is a preponderance of tumor cells (Figures 2d and 3d), SV40 might be detectable by less sensitive methods, such as Southern blotting. We are presently trying to select such specimens to test this hypothesis.

The possibility that not all of the tumor cells in a mesothelioma biopsy may express Tag is intriguing. As indicated in the previous paragraphs, tissue culture experiments indicated that usually the continuous expression of Tag is required for the maintenance of the transformed phenotype (reviewed in Ozer *et al*., 1996). However, as discussed, recent results suggest that in human cells in tissue culture (Moorwood, 1996), and in tumors developing in SV40-transgenic mice (Maroulokau *et al*., 1994; Ewald *et al*., 1996) transient expression of Tag is sufficient to induce transformation. Therefore, while we would expect that most tumor cells should express Tag, it cannot be excluded that some do not. This, together with the small number of tumor cells compared to stromal cells present in many mesothelioma specimens (Figures 2 and 3), may help in understanding why very sensitive techniques, such as PCR, are required to detect SV40.

Figures 2 and 3 are also useful for understanding the complexity of studies involving human tumors when compared to *in vitro* analyses. This complexity is more characteristic of mesothelioma, a malignancy originating from a monolayer of cells infiltrating the underlying parenchyma, with both an epithelial and a sarcomatous component, the latter almost indistinguishable from reactive stromal cells unless special stains are performed (Figure 3). Some questions which are obvious for tissue culture experiments, such as the number of malignant cells studied, the copy number of SV40 per cell, the amount of Tag per malignant cell, or the molar ratio of Tag and p53 in malignant cells, etc., are not that obvious when dealing with an heterogeneous human tumor; rather, there are many potential pitfalls if one tries to apply the logic of *in vitro* experiments to human mesothelioma.

In conclusion, the complexity of these human tumors, and the possibility that not all of the tumor cells express Tag may account for the requirement of the PCR, to demonstrate SV40 in humans.

Possible Clinical Implications and Future Directions

SV40 is an oncogenic virus capable of transforming human cells. Therefore, the presence of SV40 or related DNA sequences in human cells should not be an healthy event. On the other hand, the presence of SV40 sequences and the expression of a Tag-like protein in human tumors do not establish a cause-effect relationship. Viral sequences could represent an incidental finding with no relationship with the malignant phenotype. In support of this hypothesis, the non-homogeneous expression of Tag in tumor cells may suggest that mesotheliomas might be polyclonal in origin. In this event, the expression of Tag should be dispensable for carcinogenesis. It is also possible that SV40 infects mesothelial cells following tumor development, but this seems unlikely because SV40 sequences have been recently detected in reactive human mesothelial cells (i.e., hyperplastic but not malignant cells) suggesting that SV40 for unknown

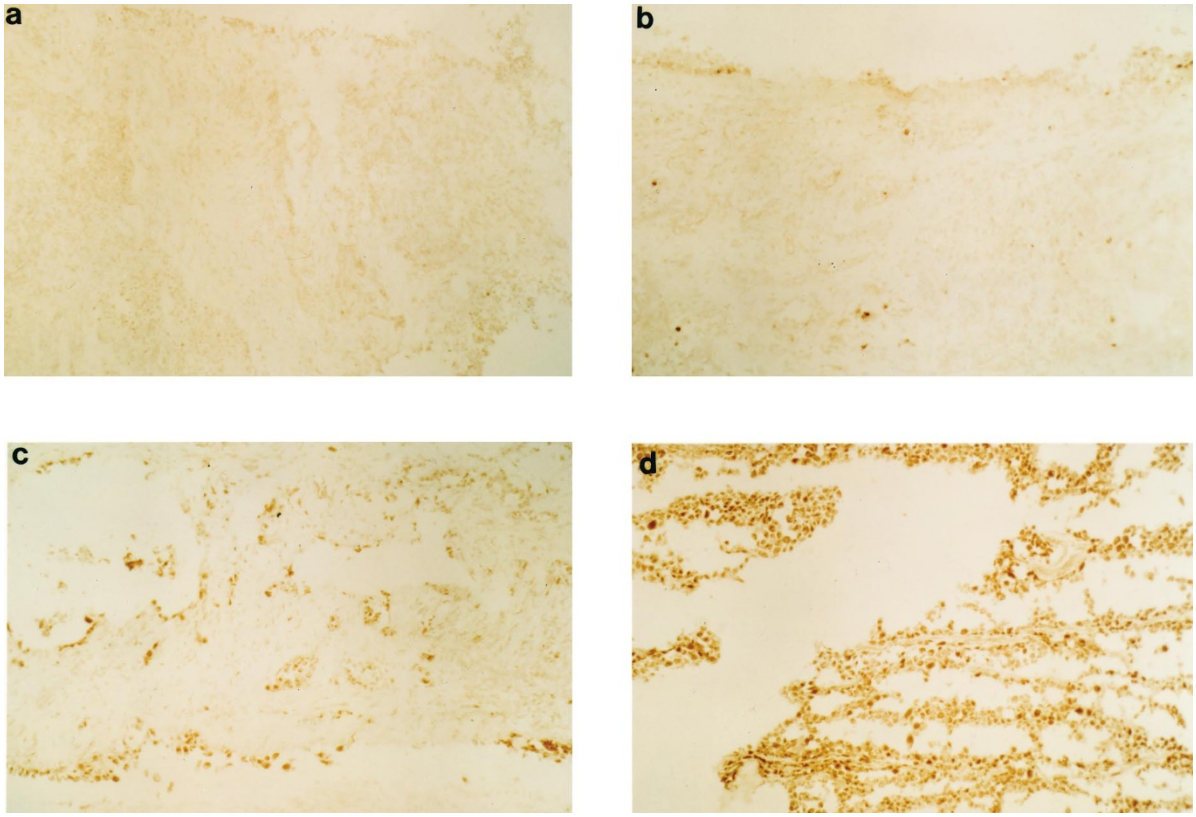

Figure 2 Immunostaining for Tag of human mesothelioma. (**a**) sample negative for Tag staining; (**b**) sample with less than 25% of cells positive for Tag; (**c**) sample with less than 50% of cells positive for Tag; (**d**) sample with more than 50% of cells positive for Tag. The anti-Tag used was pAb419 (Oncogene Science); Magnification 40 ×

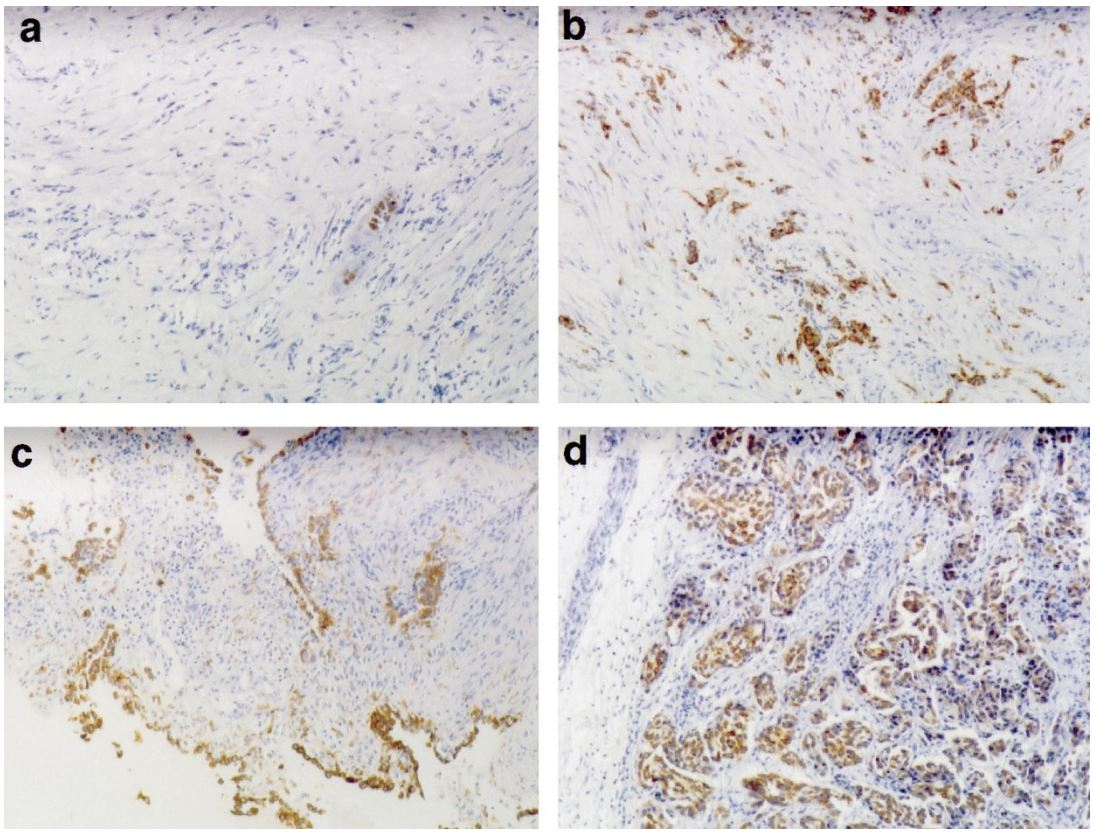

Figure 3 Cytokeratin stain of human mesothelioma. (**a**) nest of malignant cells (cytokeratin positive) in a background of reactive non-malignant stromal cells (cytokeratin negative); (**b**) this sample contains less than 25% of malignant cells (cytokeratin positive); (**c**) this sample contains less than 50% of malignant cells (Cytokeratin positive); (**d**) this sample contains more than 50% of malignant cells (cytokeratin positive). Cytokeratin stains mesothelial cells (brown staining) but it does not stain stromal cells. Because epithelial cells are also stained with cytokeratin, this marker alone cannot be used to distinguish mesothelioma from metastatic carcinoma to the pleura. Magnification 20 ×

reasons targets mesothelial cells (Galateau-Sallè, 1997). In summary, the available data are still insufficient to indicate what, if any, pathogenic role SV40 and SV40-like sequences may have when detected in the tumor cells of a given patient. Furthermore, the epidemiological data available about tumors developing in recipients of SV40-contaminated vaccines are mostly incomplete. However, the SEER data available in the US since 1973 suggest that among the tumor types found to contain SV40-like sequences there has been a 20% increase since 1973 in the incidence of ependymomas and bone tumors. The incidence of mesotheliomas has increased dramatically, presumably because of asbestos exposure. The contribution, if any, of SV40 to the increased incidence of mesotheliomas is presently unclear. SV40 preferentially induces mesothelioma in hamsters, however, the biology of SV40 infection is different in hamster and human cells. We (Carbone *et al*., in press) and others (De Luca *et al*., in press) found that Tag is bound to p53 and Rb-related proteins in mesotheliomas, suggesting that SV40 may contribute to the development of these tumors. However, additional studies are required to determine if a sufficient amount of Tag is produced to completely inhibit the activity of these cellular tumor suppressor genes, and to determine the importance of the inactivation of these tumor suppressors for the development of mesotheliomas. It has been suggested that SV40 is not a complete carcinogen in human mesothelial cells (Reddel *et al*., 1989), and we found that patients who expressed SV40-Tag contained asbestos in their lungs (Carbone *et al*., 1994). Tag-mediated inactivation of p53 may render mesothelial cells more susceptible to the transforming effects of asbestos; or Tag may contribute to the fully transformed phenotype of cells previously damaged by asbestos. A synergistic effect between asbestos and SV40 was suggested by the finding that asbestos facilitates transformation of cells in culture by SV40 (Dubes, 1993). If proven *in vivo*, such a mechanism could explain why people only exposed to asbestos or only infected with SV40 rarely develop mesotheliomas. Marsella *et al*., in press, found that p53-deficient mice are more susceptible to the induction of mesothelioma by asbestos. These results indicate that p53 plays a role in the development of mesothelioma (at least in mice) and suggest that Tag by binding p53 may potentiate the carcinogenicity of asbestos. Studies in our laboratories are in progress to evaluate the possibility that asbestos and SV40 are co-carcinogens. In conclusion, the hypothesis that SV40 may play a role in the development of human mesothelioma, or other tumors, should be carefully investigated. Regardless of the role that SV40 might have in the development of some malignancies, and even if SV40 had absolutely no role in their development, SV40 sequences and Tag-like expression in human tumors may represent useful targets for either diagnostic or therapeutic purposes. For example, the histologic diagnosis of mesothelioma is a diagnosis of exclusion, because there are not specific markers for mesothelioma, because mesotheliomas may resemble metastatic lung and breast carcinomas, and because these tumor types share several immunohistochemical markers (Figure 3). Because SV40-like sequences have not been detected in carcinomas, if present, these sequences may represent a useful marker to confirm the diagnosis of mesothelioma.

Furthermore, the expression of Tag by malignant cells could be exploited for specific immunotherapeutic approaches. Tag is mainly a nuclear antigen, from which Tag peptides are regularly generated and presented by MHC class I molecules to cytolytic T cells (Tevethia, 1990; Bright *et al*., 1994). Extensive work in rodents has shown that protective immunity to SV40 Tag can be induced (Tevethia, 1990). The presence of Tag-like protein on tumor cells, and its absence from the surrounding normal tissue (Bergsagel *et al*., 1992; Carbone *et al*., 1994; and Figure 2), raises the possibility of an immunotherapeutic approach for these malignancies (at least in the portion of patients that express Tag). Experiments in collaboration with Martin W. Kast to design new diagnostic and therapeutic approaches are currently in progress in our laboratories.

Acknowledgements

We thank Drs Susan Fisher, Robert L Garcea, Martin W Kast, John A Lednicky, Umberto Saffiotti and Luis Villarreal for critical reading of this manuscript. This work was supported by the National Cancer Institute grant CA-74283-01 (to MC).

References

Appel JD, Fasy TM, Kohtz DS, and Johnson EM. (1988). *Proc. Natl. Acad. Sci. USA*, **85,** 7670–7674.

Avantaggiati ML, Carbone M, Graessman A, Nakatani Y, Howard B, and Levine AS. (1996). *EMBO J.*, **15,** 2236–2248.

Banga SS, Kim S, Hubbard K, Dasgupta T, Jha KK, Patsalis P, Hauptschein R, Gamberi B, Dalla-Favera R, Kraemer P, and Ozer HL. (1997). *Oncogene*, **14,** 313–321.

Bell DW, Jhanwar SC, & Testa JR. (1997). *Proceedings of the IV International Mesothelioma Conference*. University of Pennsylvania, Philadelphia PA, May 13–15. G4, pp. 27.

Bergsagel DJ, Finegold MJ, Butel JS, Kupsky WJ, and Garcea R. (1992). *N. Engl. J. Med.*, **36,** 988–993.

Bikel I, Montano X, Agha ME, Brown M, McCormack M, Boltax J, and Livingston DM. (1987). *Cell*, **48,** 321–330.

Bright RK, Shearer MH, and Kennedy RC. (1994). *Clin. Exp. Immunol.*, **96,** 491–499.

Carbone M, Lewis AM Jr, Matthews BJ, Levine AS, and Dixon K. (1989). *Cancer Res.*, **49,** 1565–1571.

Carbone M, Pompetti F, Cicala C, Nguyen F, Dixon K, and Levine AS. (1991). In: *Molecular basis of human cancer*, Nicolini C (ed.), Plenum Publishing Co New York, pp. 191–206.

Carbone M, Pass HI, Rizzo P, Marinetti MR, Di Muzio M, Mew DJY, Levine AS, and Procopio A. (1994). *Oncogene*, **9,** 1781–1790.

Carbone M, Rizzo P, Procopio A, Pass HI, Gebhardt MC, Mangham C, Hansen M, Malkin DF, Bushart G, Pompetti F, Picci P, Levine AS, Bergsagel JD, and Garcea RL. (1996). *Oncogene*, **13,** 527–535.

Carbone M, Rizzo P, Grimley PM, Procopio A, Mew DJY, de Bartolomeis A, Esposito V, Shridar V, Giuliano MT, Steinberg SM, Levine AS, Giordano A, and Pass HI. *Nature Med.*, In press.

Cicala C, Pompeti F, Nguyen P, Dixon K, Levine AS, and Carbone M. (1992). *Virology*, **190,** 475–479.

Cicala C, Pompetti F, & Carbone M. (1993). *Am. J. Pathol.*, **142,** 1524–1533.

Cicala C, Avantaggiati ML, Graessmann A, Rundell K, Levine AS, and Carbone M. (1994). *J. Virol.*, **68,** 3138–3144.

Cole NC. (1996). In: *Virology*. Fields BN, Knipe DM, Howley PM (eds), Lippincott-Raven Publishers: Philadelphia, New York, vol. 2, pp. 1997–2025.

Communicable Disease Center, Report NO. 248 (1961).

Cristaudo A, Vivaldi A, Sensales G, Guglielmi G, Ciancia E, Elisei R and Ottenga F. (1995). *J Env. Pathol. Toxicol. Oncol.*, **14,** 29–34.

De Luca A, Baldi A, Esposito V, Howard CM, Bagella L, Rizzo P, Caputi M, Pass HI, Giordano GG, Baldi F, Carbone M amd Giordano A. *Nature Med.* In press.

De Villers E. (1997). *SV40: A possible human polyomavirus workshop*. National Institutes of Health, Bethesda, MD. 27–28 Jan. 1997. Transcripts. SAG, Corp. Washington DC 20008. (vol. 1) pp. 176–180.

Diamandopoulous GT. (1972). *Science*, **176,** 73–75.

Dixon K, Ryder BJ, and Burche-Jaffe E. (1982). *Nature*, **296,** 672–675.

Dubes GR. (1993). *Proc. Am. Assoc. Cancer Res.*, **34,** 185 (abs.)

Eddy BE, Borman GS, Berkeley WH, and Young RD. (1961). *Proc. Soc. Exptl. Biol. Med.*, **107,** 191–197.

Eddy BE, Borman GS, Grubbs GE and Young RD. (1962). *Virology*, **17,** 65–75.

Ewald D, Li M, Efrat S, Auer G, Wall RJ, Furth PA, and Hennighausen L. (1996). *Science*, **273,** 1384–1387.

Fanning E and Knippers R. (1992). *Annu. Rev. Biochem.*, **61,** 55–85.

Fraumeni J, Ederer F and Miller RW. (1963). *JAMA.*, **185,** 713–718.

Frost JA, Alberts AS, Sontag E, Guan K, Mumby MC, and Feramisco JR. (1994).*Mol. Cell. Biol.*, **14,** 6244–6252.

Galateau-Sallè F. (1997). *SV40: A possible human polyomavirus workshop*. National Institutes of Health, Bethesda, MD. 27–28 Jan. 1997. Transcripts. SAG, Corp. Washington DC 20008. (vol. 1) pp. 186–188.

Geissler E. (1990). *Prog. Med. Virol.*, **37,** 211–222.

Griffiths D, and Weiss R. (1997). *SV40: A possible human poliomavirus workshop*. National Institutes of Health, Bethesda, MD. 27–28 Jan. 1997. Transcripts. SAG, Corp. Washington DC 20008. (vol. 1) pp. 171–176.

Ilyinskii PO, Daniel MD, Horvath CJ, and Desrosiers RC. (1992). *J. Virol.*, **66,** 6353–6360.

Jensen F, Koprowski H, Pagano JS, Ponten J, and Ravdin RG. (1964). *J. Natl. Cancer Inst.*, **32,** 917–932.

Kirschstein RL, and Gerber P. (1962). *Nature*, **195,** 299–300.

Krieg P, Amtmann E, Jonas D, Fisher H, Zang K, and Sauer G. (1981). *Proc. Natl. Acad. Sci. USA*, **78,** 6446–6450.

La Bella F and Ozer HI. (1985). *Virus Res.*, **2,** 329–343.

Lednicky JA, Garcea RL, Bergsagel DJ and Butel JS. (1995a). *Virology*, **212,** 710–717.

Lednicky JA, Wong C and Butel JS. (1995b). *Virus Res.*, **35,** 143–153.

Lednicky JA, Stewart AR, Jenkins JJ, Finegold MJ and Butel JS. *Int. J. Cancer*, In press.

Lewis, AM Jr. (1973). In: *Biohazards in Biological Research*. Hellman A, Oxman M, and Pollack R (eds.), Cold Spring Harbor Lab Press, New York, NY, pp.96–113.

Lewis AM Jr and Martin RG. (1979). *Proc. Natl. Acad. Sci. USA*, **76,** 4299–4302.

Lill NL, Tevethia MJ, Eckner R, Livingston DM, and Modjtahedi N. (1997). *J. Virol.*, **71,** 129–137.

Linnainmaa K. (1997). *Proceedings of the IV International Mesothelioma Conference*. University of Pennsylvania, Philadelphia PA, May 13–15. S3, pp. 30.

Mark EJ, & Yokoi T. (1991). In: *The third wave of asbestos disease: exposure of asbestos in place*. Landrigan PJ, and Kazemi H. (eds.), *Ann. N.Y. Acad. Sci.*, **643,** 196–204.

Maroulakaou IG, Anver M, Garrett L and Green JE. (1994). *Proc. Natl. Acad. Sci. USA*, **91,** 11236–11240.

Marsella JM, Liu BL, Vaslet CA, and Kane AB. *Environmental Health Perspectives*. In press.

Marshall J, Smith AE and Cheng SH. (1991). *Oncogene*, **6,** 1673–1676.

Martini F, Iaccheri L, Lazzarin L, Carinici P, Corallini A, Gerosa M, Iuzzolino P, Barbanti-Brodano G, and Tognon M. (1996). *Cancer Res.*, **56,** 4820–4825.

Melnick JL, & Stinbaugh S. (1962). *Proc. Soc. Exptl. Biol. Med.*, **109,** 965–968.

Melnick JL. (1996). In: *Virology*. Fields BN, Knipe DM, Howley PM (eds), Lippincott-Raven Publishers, Philadelphia, New York, vol. 1, pp. 655–712.

Monini P, de Lellis L and Barbanti-Brodano G. (1995). In: *DNA tumor viruses oncogenic mechanisms*, Barbanti-Brodano G, Bendinelli M, and Frieman H (eds.), Plenum Publishing Co. New York, NY pp. 51–73.

Moorwood K, Price TNC and Mayne LV. (1996). *Exp. Cell Res.* **223,** 308–313.

Morris JA, Johnson KM, Aulisio GC, Chanock RM and Knight V. (1961). *Proc. Soc. Exptl. Biol. Med.*, **108,** 56–64.

Mortimer EA, Lepow ML, Gold E, Robins FC, Burton GJ and Fraumeni JF. (1981). *N. Engl. J. Med.*, **305,** 1517–1518.

Olin P. (1997). *SV40: A possible human polyomavirus workshop*. National Institutes of Health, Bethesda, MD. 27–28 Jan. 1997. Transcripts. SAG, Corp. Washington DC 20008. (vol. 1) pp. 315–323.

Ozer HL, Banga SS, Dasgupta T, Houghton JM, Hubbard K, Jha KK, Kim SH, Lenhan M, Pang Z, Pardinas JR and Patsalis PC. (1996). *Exp. Gerontol.* **31,** 303–310.

Pass HI, Kennedy RC and Carbone M. (1996). In: *Important advances in Oncology*, Rosenberg SA, Hellman S, and De Vita T (eds.), Lippincott-Raven Publishers, Phildelphia PA, pp. 89–108.

Pass HI. (1997). *SV40: A possible human polyomavirus workshop*. National Institutes of Health, Bethesda, MD. 27–28 Jan. 1997. Transcripts. SAG, Corp. Washington DC 20008. (vol. 1) pp. 180–185.

Pepper C, Jasani B, Navabi H, Wynford-Thomas D and Gibbs AR. (1996).*Thorax*, **51,** 1074–1076.

Peto J, Hodgson JT, Matthews FE and Jones JR. (1995). *The Lancet*, **345,** 535–539.

Price B. (1997). *Am. J. Epidemiol*, **145,** 211–218.

Rabson AS, O'Connor GT, Kirschstein RL and Branigan WJ. (1962). *J. Natl. Cancer Inst*, **29,** 766–787.

Ray AF. (1995). In: *DNA tumor viruses oncogenic mechanisms*, Barbanti-Brodano G, Bendinelli M and Frieman H (eds.), Plenum Publishing Co. New York, NY pp. 15–26.

Ray AR, Grossman SA, Ronnett BM, Bigner SH, Vogelstein B amd Shah K. (1994). *J. Neuro-Oncology*, **20,** 55–58.

Reddel RR, Malan-Shibley L, Gerwin BI, Metcalf RA, and Harris CC. (1989). *J. Natl. Cancer Inst.*, **81,** 945–948.

Rhim JS, Fujita J, Arnstein P and Aaronson SA. (1986). *Science*, **232,** 385–388.

Roggli VL, Sanfilippo F and Sheolburne J. (1992). In: *Pathology of asbestos and associated diseases*. Roggli VL, Geenberg SD, and Pratt PC. (eds.), Little Brown, Boston pp. 383–391.

Ruley EH. (1996). In: *Important advances in Oncology*, Rosenberg SA, Hellman S and De Vita T. (eds.), Lippincott-Raven Publishers, Phildelphia PA, pp. 89-108.

Shah KV and Nathanson N. (1976). *Am. J. Epidemiol.*, **93,** 291–297.

Shein H and Enders JF. (1962). *Proc. Natl. Acad. Sci. USA*, **48,** 1164–1172.

Simmons DT. (1995). In: *DNA tumor viruses oncogenic mechanisms*, Barbanti-Brodano G, Bendinelli M, and Frieman H (eds.), Plenum Publishing Co. New York, NY. pp. 27–50.

Soriano F, Shelburne CE and Gokcen M. (1974). *Nature*, **249,** 421–424.

Stoian M, Suru M, Iosipengo M, Athanasiu P, Nastac E, Arseni C and Alexianu D. (1984). *Rev. Roum. Med. Virol.*, **35,** 127–132.

Strickler HD. (1997). *SV40: A possible human polyomavirus workshop*. National Institutes of Health, Bethesda, MD. 27–28 Jan. 1997. Transcripts. SAG, Corp. Washington DC 20008. (vol. 1) pp. 323–340.

Strickler HD, Goedert JJ, Fleming M, Travis WD, Williams AE, Rabkin CS, Daniel RW and Shah KV. (1996). *Cancer Epidemiology, Biostatistics and Prevention*. **5,** 473–475.

Sweet BH and Hilleman MR. (1960). *Proc. Soc. Exptl. Biol. Med.*, **105,** 420–427.

Tevethia SS. (1990). *Mol. Biol. Med.*, **7,** 83–96.

Tieman F, Zerrhan J and Deppert W. (1995). *J. Virol.*, **69,** 6115–6121.

von der Weth A and Deppert W. (1992). *Virology*, **189,** 334–339.

Walsh JW, Zimmer SG and Perdue ML. (1982). *Neurosurgery*, **10,** 643–662.

Weiner LP, Herndon RM, Narayan O, Johnson RT, Shah K, Rubinestein LJ, Preziosi TJ and Conley FK. (1972). *N. Engl. J. Med.*, **305,** 1517–1518.

Weiss AF, Portmann R, Fisher H, Simon J and Zang KD. (1975). *Proc. Natl. Acad. Sci. USA*, **72,** 609–613.