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The Relative Role of Viral Transformation and Specific Cytogenetic Changes in the Development of Murine and Human Lymphomas

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This talk will be limited to a consideration of lymphoma and leukemia development (or certain types) in mice and men where there is extensive evidence for the role of the specific genetic changes recognizable at the chromosomal level. To start with the conclusion, it is clear that lymphoma development can be initiated by a variety of agents. In all probability, the initiation process creates long-lived preneoplastic cells, which are frozen in their state of differentiation and capable of continued division. These cells constitute the raw material for the subsequent cytogenetic evolution that converges towards a common, distinctive pattern. The nature of this pattern as it appears in the overt lymphomas depends on the subclass of the target lymphocyte rather than on the initiating ("etiologic") agent.

A. Human Lymphomas

The most extensive evidence concerns Burkitt lymphoma (BL). About 97% of the BLs tested that arose in the high endemic regions of Africa were monoclonal proliferations of Epstein-Barr virus(EBV)-carrying cell clones of B lymphocyte origin (Klein 1975; Klein 1978; Zur Hausen et al. 1970). BL tumor cells in vivo and derived cell lines are similar in carrying multiple copies of the EBV genome and often carry around 30–40 per cell. Some of the EBV genome copies are integrated with the cellular DNA, while the majority are present as free plasmids (Kaschka-Dierich et al. 1976; Falk et al. 1977). BL cells show no detectable viral expression in vivo except the EBV-determined nuclear antigen, EBNA (Reedman and Klein 1973), which is a DNA-binding protein that is present in all cells carrying EBV DNA. Superficially at least the properties of EBNA resemble those of the tumor (T) antigens induced by the oncogenic papovarivurses (Klein et al., to be published; Luka et al. 1978). In the majority of the cases, BL-derived cell lines arise by the growth in vitro of the same clone that is tumorigenic in vivo (Fialkow et al. 1971; Fialkow et al. 1973). These cell lines are also similar to the tumor in vivo with regard to EBNA expression. In addition, many lines (termed producers) also contain a small number of cells that switch on viral production; other lines are nonproducers (Nadkarni et al. 1969).

The EBV-carrying lymphoid cell lines with an essentially similar EBV DNA status and viral gene expression can also be derived from the peripheral blood (Diehl et al. 1968) or the lymph nodes (Nilsson et al. 1971) of normal seropositive donors; they are referred to as lymphoblastoid cell lines (LCLs). LCLs differ from BL lines in a number of phenotypic characteristics (Nilsson and Pontén 1975). On the basis of the limited information now available it has not been possible to attribute this to differences in the viral genome or the virus-cell relationship (for review see Adams and Lindah 1974). The cytogenetic differences between LCLs and BL lines discussed below suggest, on the other hand, that the differences may be determined by the cellular genome rather than by the viral genome.

There is firm evidence that EBV is a transforming virus in vitro (Gerber and Hoyer 1971; Henle et al. 1967; Miller 1971; Moss and Pope 1972) and induces lethal lymphoproliferative disease in certain nonhuman primates in vivo (Frank et al. 1976). In humans, primary infection of adolescents or young adults causes infectious mononucleosis, a self-

limiting benign lymphoproliferative disease (for review see Henle and Henle 1972). During mononucleosis a relatively small number of EBV-carrying B blasts appear in the peripheral circulation; they disappear again during convalescence (Klein et al. 1976). They are probably reduced in number by the EBV-specific killer T cells that appear in parallel. The killer cells can lyse autologous and allogeneic EBV-carrying (but not EBV-negative) target cells without any apparent syngeneic restriction (Bakacs et al. 1978; Jondal et al. 1975; Svedmyr & Jondal 1975; Svedmyr et al. 1978). In fatal cases of mononucleosis the lymphoid tissues are usually infiltrated with EBNA-positive cells (Britton et al. 1978; Miller, personal communication). In some acute cases of infectious mononucleosis EBV DNA could be demonstrated in the bone marrow during the acute phase of the disease (Zur Hausen 1975). Infectious mononucleosis is thus accompanied by, and probably due to, an extensive but usually temporary proliferation of EBV-carrying cells. Moreover, it has been postulated that a number of chronic mononucleosis like conditions, which border on lymphoma and are often familiar and X-linked, are due to polyclonal proliferation of EBV-carrying cells which is not properly immunoregulated (Purtilo et al. 1978).

As already mentioned, experimental oncogenicity of EBV is restricted to a few New World monkey species (Frank et al. 1976). Large apes and Old World monkeys are resistant. This is understandable, because they carry EBV-related herpesviruses that induce cross-neutralizing antibodies. The EBV-like chimpanzee, baboon, and orangoutan viruses were studied in some detail (Falk et al. 1977; Gerberg et al. 1976; Ohno et al. 1977; Rabin et al. 1978). They can immortalize B lymphocytes. Their DNA sequences are partially homologous with EBV and their antigens are crossreactive but not identical.

New World monkeys tested carried no EBV-related virus and had no cross-neutralizing antibodies. Some of them have lymphotropic herpesviruses of their own (reviewed by Deinhardt et al. 1974), but these are quite different from the EBV family and will not be discussed here.

The nature of the EBV-induced malignant lymphoproliferative disease in susceptible New World monkeys (e.g., marmosets) has not been analyzed in detail. It is not yet clearly established whether it is due to the polyclonal growth of virally transformed cells like the rare fatal cases of human mononucleosis or is a monoclonal tumor like BL.

Parallel cytogenetic and nude mouse inoculation studies (Nilsson et al. 1977; Zech et al. 1976) have recently dispelled the earlier notion that all EBV-transformed human lines are tumorigenic irrespective of origin. Virally immortalized normal B lymphocytes remained purely diploid during several months of cultivation in vitro, failed to grow subcutaneously in nude mice, and had a low (1%–3%) cloning efficiency in agarose. After prolonged passage in vitro they became aneuploid as a rule and acquired the ability to grow in nude mice and in agarose. In contrast, BL biopsy cells and derived lines were aneuploid and tumorigenic from the beginning and had a high clonability in agarose. In immunologically privileged sites such as the nude mouse brain or the subcutaneous tissue of the newborn nude mouse, both diploid LCL and aneuploid BL lines could grow progressively, however (Giovanella et al. 1979). Growth in these immunologically privileged sites did not enable the diploid LCL to grow subsequently in the subcutaneous tissue of the adult nude mouse, however.

The chromosomal changes of the long-passaged LCLs showd no apparent specific features. In contrast, most BL cells contain the same highly specific marker. The marker was first identified as 14q+, with an extra band at the distal end of the long arm of one chromosome 14 (Manolov and Manolova 1972). 14q+ markers were subsequently described in a variety of other lymphoreticular noeplasias (Fleischman and Prigogina 1977; Fukuhuara and Rowley 1978; Mark et al. 1977; McCaw et al. 1977; Mitelman and Levan 1973; Yamada et al. 1977; Zech et al. 1976). Closer scrutiny revealed important differences between the 14q+ marker of BL and non-BL lymphomas. In BL the extra band is derived from chromosome 8 (Zech et al. 1976) and represents a reciprocl translocation between 8 and 14 with precisely identical breaking points in different cases (Manolova et al. 1979). In non-BL with a 14q+ marker the donor chromosome was variable; pieces could be derived from chromosomes 1, 4, 10, 11, 14, 15 or 18 in addition to 8 (reviewed by Fukuhara and Rowley 1978).

The BL-associated reciprocal 8;14 translocation is not limited to EBV-carrying African

BL. It was also found in EBV-negative American BL (McCaw et al. 1977; Zech et al. 1976) and in the rare B-cell form of acute lymphocytic leukemia (Mitelman et al. 1979) believed to represent the neoplastic growth of the same cell type as BL. This, together with the fact that EBV-transformed LCLs of non-BL origin do not carry the 8;14 translocation, suggests that EBV is not involved in causing the translocation.

We have suggested (Klein 1978) that African BL develops in at least three steps. The *first step* is the EBV-induced immortalization of some B lymphocytes upon primary infection. This does not differ from the seroconversion of normal EBV carriers, except perhaps in one respect. The prospective study in the high endemic West Nile district has suggested that pre-BL patients may carry a higher load of EBV-harboring cells than normal controls (de Thé et al. 1978). The *second step* is brought about by an environment-dependent factor, perhaps chronic holoendemic malaria (Burkitt 1969; O'Connor 1961), that would urge the latent EBV-carrying cells frozen at a particular stage of B-cell differentiation to chronic proliferation and could further facilitate this process by a relative immunosuppression. In a way this would resemble the promotion step in experimental two-phase carcinogenesis. By forcing the long-lived preneoplastic cells to repeated division, the environmental cofactor would provide the scenario for cytogenetic diversification. The *third and final step* would occur when the "right" reciprocal 8;14 translocation occurred; this would lead to the outgrowth of an autonomous monoclonal tumor.

The reciprocal translocation could arise by a purely random Darwinian process or by more specific mechanisms as suggested by Fukuhara and Rowley (1978). The ubiquity of EBV, the high virus load carried by the African populations at risk, and the large number of cell divisions that must occur in the chronically hyperplastic lymphoreticular system of the parasite-loaded children makes a purely random process perfectly conceivable, particularly when contrasted against the relative rarity of the disease even in the high endemic regions.

The majority of the sporadic cases in nonendemic areas (Andersson et al. 1976), which show no evidence of clustering, are constituted by EBV negative BLs. The identical 8;14 translocation suggests that their development is triggered by the same final cytogenetic event, while the earlier initiating and promoting steps are probably quite different. Initiation may be due to another viral or nonviral agent or could reflect a spontaneous (mutation-like?) change.

The frequent involvement of chromosome 14 in the genesis of human neoplasia of largely, if not exclusively, B-cell origin suggests that some determinant(s) on this chromosome is (are) closely involved with the normal responsiveness of the B lymphocyte to growth-controlling mechanisms. It is interesting to note that chromosome 14 anomalies were found in a high frequency in ataxia teleangiectasia, a condition noted for a marekdly increased incidence of lymphoreticular neoplasia (McCaw et al. 1975). It must be noted, however, that the most frequent breakpoint in chromosomes of patients with ataxia telangiectasia is in band 14q12, whereas the BL-associated breakpoint is in band 14q32.

B. Murine T Cell Leukemia

Dofuko et al. (1975) reported that the cells involved in "spontaneous" T cell leukemias of the AKR mouse frequently contain 41 chromosomes insted of 40, with trisomy of chromosome 15 as the most common change. We found a similar predominance of trisomy 15 in T cell leukemias induced in C57BL mice by two different substrains of the radiation leukemia virus (Wiener et al. 1978a,b) and by the chemical carcinogen dimethylbenz(a)anthrancene (Wiener et al. 1978c). Trisomy 17 was the second most common anomaly, much less frequent than trisomy 15, and never found without the latter. Trisomy 15 was also identified as the main cytogenetic change in X-ray-induced mouse lymphomas (Chang et al. 1977). In contrast, lymphoreticular neoplasias of non-T cell origin, induced by the Rauscher, Friend, Graffi, and Duplan viruses, some B lymphomas of spontaneous origin, and a series of mineral oilinduced plasmacytomas showed no trisomy 15 (Wiener et al., unpublished data). The question whether they have other types of distinctive chromosomal changes has not yet been answered.

It is sometimes postulated that all murine T cell lymphomas are due to the activation of latent type C viruses. Careful examination of the pathogenesis of these lymphomas makes

this most unlikely, however (for review see Haran-Ghera and Peled 1979). It is more likely that X-rays and chemical and viral carcinogens can all play the role of initiating agents that can create long-lived preleukemic cells. The development of overt leukemia depends on additional changes that occur during the prolonged latency of the preleukemic cells in their host. It is very likely that the duplication of certain gene(s), reflected by the trisomy 15, plays a key role in this process.

The trisomy of the spontaneous AKR leukemia is particularly remarkable in this context. The high leukemia incidence of this strains stems from prolonged inbreeding and selection for leukemia. As already mentioned in the first part of this article, AKR mice carry at least four different genetic systems that favor leukemia development by independent mechanims (for review see Lilly and Pincus 1973). In spite of this high genetic preneness for leukemia, the disease fails to appear until 6–8 months after birth. This long latency period, together with the appearance of trisomy 15 in overt leukemia, supports the notion that the leukemogenic virus is not self-sufficient in changing normal T lymphocytes to autonomous leukemia cells.

Is there a specific region on chromosome 15 that needs to be duplicated for the development of leukemia? We have also examined the karyotype of dimethylbenz(a)anthracene-induced T cell leukemias in CBAT6T6 mice (Wiener et al. 1978a,b). The T6 marker has arisen by a breakage of chromosome 15 not far from the centromere and translocation of the distal part of the long arm to chromosome 14. Six independently induced leukemias showed trisomy of the 14;15 translocation, while the small T6 marker was present in only two copies. This suggests the involvement of specific region(s) in leukemogenesis localized in the distal part of the long arm of chromosome 15. Additional translocations will be helpful in defining the region more precisely.

C. Is Trisomy a Cause or a Consequence of a Murine T-Cell Leukemia?

It is conceivable that trisomy 15 is merely a consequence of leukemogenesis. It could be imagined, for example, that it is only one among many different trisomies that can arise but that the others are incompatible with continued life and proliferation of the murine T-lymphocytes. We have recently excluded this possibility by inducing leukemias in mice that carry Robertsonian translocation (Spira et al., to be published). T-cell leukemias were induced by the chemical carcinogen DMBA and by Moloney virus, respectively, in mice carrying 1;15, 5;16, and 6;15 Rb translocations. In the resulting leukemias the entire translocation chromosome was present in three copies. This proves that trisomy of even the longest chromosome (No. 1) must be tolerated by the cell if it is fused with the crucially important chromosome 15. This strongly supports the idea that trisomy of chromosome No. 15 is essential for T-cell leukemogenesis.

Our most recent studies (Wiener et al., to be published) have focused on the induction of T-cell leukemias in F_1 hybrids derived from crosses between mouse strains with cytogenetically distinguishable 15-chromosomes. The CBAT6T6 strain that carries the characteristic 14;15 translocation was crossed with strains AKR, C57Bl, and C3H, all of which have cytogenetically normal 15-chromosomes. T-cell leukemias were induced in the resulting F_1 hybrids by DMBA and Moloney virus, respectively. Duplication of chromosome 15 was nonrandom, depending on the genetic content of the chromosome. In the crosses between T6T6 and AKR, the AKR-derived normal 15 chromosome was duplicated preferentially. Both the C57Bl×T6T6 and C3H×T6T6 F_1 hybrids showed the opposite behavior, with preferential duplication of the T6-derived 14;15 translocation chromosome. Since the chances for duplication must be approximately equal for the 15 chromosomes derived from one or the other parental strain, this must mean that the selective advantage of the two alternative 15-duplications must be unequal in the course of leukemia development. These findings suggest a certain "hierarchy" among what is probably an allelic series of genes located on chromosome 15. Apparently, the genes are unequal with regard to the selective advantage they convey on the preleukemic cell in relation to its transition to turning into overt leukemia.

D. Is Abelson Virus a Transducer or Cellular Gene?

In contrast to all other known mouse leukemia viruses, Abelson virus transforms (immortalizes) lymphocytes in vitro and induces leukemia after short latency periods in vivo. It has been shown (Klein 1975) that the viral genome contains a large cellular insert that occupies the most of the middle portion of the viral genome. It specifies a large polyprotein that is probably associated with the cell membrane and is endowed with protein kinase activity.

We have recently examined the karyotype of Abelsonvirus induced leukemias (Klein al. 1980) and found it to be purely diploid with no demonstrable anomalies by banding analysis. Moreover, the Abelson virus transformed lines remained diploid over long periods of time.

Is it conceivable that the change in gene dosage that is achieved by the duplication of a whole chromosome in leukemias that arise after long latency periods is directly achieved by the viral transduction of a corresponding piece of crucial genetic information? If this is correct, it would follow that directly transforming viruses that carry pieces of normal genetic information and induce tumors with short latency periods would tend to induce diploid tumors.

Clearly, changes in gene dosage, whether achieved by chromosome duplication or viral transduction, must play an important role in the emancipation of tumor cells from host control.

E. Some Conclusions

The following points can be made on the basis of these findings and related findings of others.

I. Transformation In Vitro Is Not Synonymous with Tumorigenicity In Vivo

This point has been made many times before, but it can hardly be overemphasized. To mention only a few examples, Dulbecco and Vogt (1960) showed in their pioneering studies that foci of cells transformed in vitro by polyoma virus were not necessarily tumorigenic; at least one additional step was required for growth in vivo. Stiles et al. (1975) reported that human lines transformed by simian virus 40 failed to grow in nude mice in contrast to the regular takes of culture lines derived from tumors in vivo. Diploid lymphoblastoid cell lines transformed in vitro by EBV are clearly "immortal" but nontumorigenic in nude mice as already mentioned (Nilsson et al. 1977).

Transformation in vitro may merely reflect a relative emancipation of the cell from its earlier dependence on exogenous mitogenic signals. Most and perhaps all normal cells have a limited lifespan in vitro. Lymphocytes will not grow, not even temporarily, unless supplied with appropriate mitogenic factors. Transformation in vitro abolishes this requirement. It also "freezes" differentiation at a given level.

It is noteworthy that transformed fibroblasts and lymphocytes show certain common changes associated with immortalization in spite of their very different phenotypes – namely, increased resistance to saturation density, decreased serum requirements, and altered lectiyn agglutination and capping patterns (Steinitz and Klein 1975; Steinitz and Klein 1977; Yefenof and Klein 1976; Yefenof et al. 1977).

Most DNA viruses that transform in vitro induce DNA synthesis and mitosis in their target cells (Einhorn and Ernberg 1978; Gerber and Hoyer 1971; Gershon et al. 1965; Martin et al. 1977; Robinson and Miller 1975). For the oncogenic papovavirus systems it has been shown that the virally determined T-antigen or one from of it plays a direct role in initiating host cell DNA synthesis (Martin et al. 1977).

If transformation in vitro reflects a "built-in" ability to grow in the absence of exogenous stimulation, tumorigenicity in vivo must imply in addition, resistance to negative feedback regulations of the host. The latter may be brought out by appropriate cytogenetic changes. Trisomy, as observed in the murine T cell leukemias, may tilt the balance of the long-lived preneoplastic cells towards definite disobedience through gene dose effects. Reciprocal translocations that give rise to the Philadelphia chromosome and the 8;14 translocation associated with BL may also work through gene dosage – e.g., by position effects that stop the function of important regulatory genes when they are dislocated from their natural surroundings. Similar position effects may be responsible for the action of *src*, the extra genetic information carried by the transforming avian sarcoma viruses. Conceivably, this originally cell-derived information may become integrated, together with the rest of the proviral DNA, into new regions where it is no

longer subject to the same control as in the original location (Stehelin et al. 1976; Varmus et al. 1976). In this connection, our recent finding on the Abelson virus induced leukemia system may be of interest. This virus, as the only one among the known murine leukemia viruses, transforms in vitro and induces leukemia after only a short latency period in vivo. It is a highly defective virus, with a large cellular insert in its middle (Rosenberg and Baltimore 1980). Sequences homologous with the cellular insert and proteins identical or immunologically cross reactive with its product are present in normal mouse cells.

We have recently examined a series of Abelson virus induced leukemias and found them to be purely diploid (Klein et al. 1980). It is intriguing to speculate that transformation is compatible with diploidy in this case, since the provirus-mediated integration of the cell-derived sequences may alter gene dosage in a way appropriate to generate leukemia.

The apparently tissue-specific involvement of different chromosomes in tumor-associated nonrandom karyotype changes suggests that genes that are of crucial importance for the responsiveness of different cell types to growth control are located on different chromosomes. Some determinant on human chromosome 14 thus appears to be involved with the normal responsiveness of the B lymphocyte; determinants on chromosome 22 or 9 (or both) appear to influence myeloid differentiation; the dosage of some determinant on murine chromosome 15 seems to influence the balance between the restrained proliferation of the preleukemic cell and overt leukemia.

II. Host Cell Controls Can Modify the Expression of Transformation In Vitro

The successful isolation of phenotypic revertants from both chemically and virally transformed cell lines demonstrates the importance of host cell controls for the expression of transformation-associated characteristics. Sachs and his group (Yamamoto et al. 1973) have shown that specific chromosomal changes must play an important role in transformation and reversion. As a rule transformation was accompanied by the duplication of some chromosomes. On reversion, the same chromosomes often decreased in number, whereas other increased (Benedict et al. 1975; Yamamoto et al. 1973). Sachs speaks about expressor and suppressor elements and stresses the importance of their balance for the control of the normal vs the transformed phenotype. The temperature-sensitive host control mutants, isolated from virally transformed cell lines by Basilico (1977), are another important demonstration of cellular forces that can counteract the transforming function of an integrated viral genome.

III. Host Cell Controls Can Reverse Tumorigenic to Nontumorigenic Phenotypes

Tumorigenicity in vivo can be counteracted experimentally by two fundamentally different types of control, i.e., genetic and epigenetic. The former was demonstrated by somatic hybridization experiments. Fusion of tumorigenic cells with low or nontumorigenic normal or transformed partners has regularly led to a suppression of tumorigenicity as long as the hybrid has maintained a nearly complete karyotype (Harris 1971; Harris et al. 1969; Klein et al. 1971; Wiener et al. 1971). High tumorigenicity reappeared after the loss of specific chromosomes derived from the nontumorigenic partner (Jonasson et al. 1977; Wiener et al. 1971).

Suppression of tumorigenicity by normal cells was equally effective with tumors of viral, chemical, and spontaneous origin. Different types of normal cells were effective, including fibroblasts, lymphocytes, and macrophages. It is not known whether the normal karyotype compensates a deficiency of the malignant cell by genetic complementation or acts by imposing normal responsiveness to its own superimposed growth control. The latter possibility appears more likely. It could be explored by determining whether the reappearance of high tumorigenicity is linked to the loss of different chromosomes, depending on the type of normal cell used for the original suppressive hybridization.

A fundamentally different, nongenetic mechanism of malignancy suppression was discovered by Mintz, who demonstrated the normalization of diploid teratocarcinoma cells after their implantation into the early blastocyte (Mintz and Illmensee 1975). It is not yet clear whether this is a special case, dependent on the pluripotentiality of the teratocarcinoma cell and its normal karyotype, or is of more general significance. The well-documented abilities of

certain tumor cells to respond to differentiation-inducing stimuli represent more limited examples of the same or similar phenomena (Azumi and Sachs 1977; Rossi and Friend 1967).

IV. Concept of Convergence in Tumor Evolution

This concept is not new. In essence, it corresponds to one of the rules of tumor progression as formulated by Foulds (1958). He stated that the "multiple reassortment of unit characteristics" that formed the basis of the progression concept "could follow one of several alternative pathways of development." Some aspects of this process were stated here in a more specific way. They are as follows:

1. Like chemical or physical carcinogens, *viruses* play essentially the role of *initiators* in tumor progression. Their major effect is the establishment of *long-lived* preneo*plastic* cells.
2. *Specific genetic changes* are responsible for the transition of preneoplastic to frankly malignant cells. In some systems they are expressed as cytogenetically detectable chromosomal anomalies which are characteristic for the majority of the tumors that originate from the same target cell. The changes may arise by random mechanisms. They are selectively fixed due to the increased growth advantage of the clone that carries them. This advantage is based on a decreased responsiveness to growth-controlling or differentiation-inducing host singals. This selection process, rather than any specific induction mechanism, is responsible for the "cytogenetic convergence" of preneoplastic cell lineages initiated ("caused") by widely diverse agents towards the same nonrandom chromosomal change.
3. The cytogenetic changes act by shifting the balance between genes that favor progressive growth in vivo and genes that counteract it. Changes in effective gene dosage are brought about by nonrandom duplication of a whole chromosome, as in trisomy, or by reciprocal translocation that may effect gene expression on the donor or the recipient chromosome.

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