

Tumor Associations of EBV—Historical Perspectives

George Klein

Abstract This is a brief history of our collaborative work with Werner and Gertrude Henle, Francis Wiener, George and Yanke Manolov, and others on the association of Epstein-Barr virus (EBV) with Burkitt lymphoma and other human tumors. Special emphasis is put on the question where EBV is a true cancer virus.

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1 Introduction

The inspiring articles of Dennis Burkitt and Dennis Wright in the early 60s made the scientific community aware of the African childhood lymphoma prevalent in hot and humid regions of Africa and the “starry sky” like histology. The suggestion that an insect transmitted virus may cause the disease triggered researchers in numerous laboratories to look for the hypothetical agent. The search was facilitated by the fact that the tumor readily fell apart into single cell suspensions without any trypsinization and grew readily into cell lines.

The Virus Cancer Program of the NIH was in full swing. One day—probably in 1963 or 1964—I visited John Moloney, who headed the program, to tell him the latest news about our project on virus-induced mouse tumors. Tony Epstein

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was the other visitor. I knew Tony from his earlier visits to Torbjörn Caspersson's department in Stockholm where I worked. He showed EM images of cell lines from what was now called Burkitt lymphoma (BL) to John. In some lines, herpes-type virus particles could be seen in a small minority of the cells that were clearly degenerating and dying. John and I thought that the tumor cells may have picked a common herpesvirus as a passenger. But Tony said: It may be a wild goose but it is a goose that has to be chased. Right he was.

Soon thereafter, Werner and Gertrud Henle in Philadelphia performed immunofluorescence tests on the same lines and showed that the virus containing cells failed to react with antibodies against any known herpesvirus. This was, therefore, a new human herpesvirus. The Henles and we decided to call it EBV.

We were ready to join the adventure of looking for the footsteps of a virus in proliferating BL cells, using the experience we had from work with virus-induced mouse lymphomas. We were fortunate to establish an "air bridge" with Peter Clifford, Head of the ENT Department at the Kenyatta National Hospital in Nairobi. Getting in touch with him, we followed the percept that if you look for a collaborator to do a really hard job with you, find the busiest person and he will do it.

Peter was the only ENT surgeon between Johannesburg and Cairo with an immense working load, but passionately interested in BL. He has developed its chemotherapy in parallel with Dennis Burkitt. Unlike Burkitt, he gave only moderate doses to spare the immune system. The frequency of long-term survivors—or, as it turned out later, cures—was higher in his material than in Burkitt's more drastically treated patients.

On my request for biopsies and sera, the material started coming with clock-work regularity every Tuesday, with the only weekly SAS plane from Nairobi, frozen sera in dry ice, live tumor tissue in wet ice, in great abundance. Every Tuesday night was Burkitt night at our laboratory for about ten years. In addition to what we did with the material, we also fanned it out to other laboratories in Europe, Japan, and the USA.

Our most significant finding was the discovery of EBNA, the EBV-encoded nuclear antigen which later turned out to be a conglomerate of six different proteins. When we first detected EBNA, it was still not clear whether the virus was present in some hidden form in the proliferating cells of the tumor that have not entered the lytic viral cycle which inevitably led to cell death.

To detect EBNA, Beverly Reedman and I departed from the observation of John Pope in Australia, showing that an EBV-specific complement fixing antigen was present in a BL line that did not make any virus. We decided to look for it by anticomplement fluorescence. EBNA soon appeared in all its magnificence (Reedman and Klein 1973).

The detection of EBNA by anticomplement fluorescence was tricky, and sometimes, it did not work. Years later, under the rule of Idi Amin, a note appeared in Newsweek saying that the African radiotherapist, Charles Olweny, Head of the Uganda Cancer Center at that time, was found in the forest with his head cut-off together with two other colleagues, because they opposed the renaming of

Makerere Medical College to Idi Amin University. I tried to call Charles. He was not there. I called a week later and he was still not there. But he was going to come the week after. When I called the third time, he came on the line. Charles! I shouted. Are you all right? No George, the EBNA test is not working, he said.

Lloyd Old and Herbert Oettgen at Sloan Kettering were among the recipient laboratories in the USA. They detected an EBV-specific soluble antigen by immunoprecipitation in BL and also nasopharyngeal carcinoma (NPC) specimens. The EBV/NPC association was confirmed by the serology of the Henles. But it was not clear whether the virus was carried by the carcinoma cells or by the abundant lymphoid infiltrate. We were inclined to blame the latter until we found that the lymphoid infiltrate of NPC consisted mostly of T cells, not known as EBV harboring cells at the time. Importantly, the EBNA test clearly showed that latent virus was carried by the carcinoma cells themselves. With Harald zur Hausen, we could also confirm the presence of multiple EBV genomes in both BL and NPC cells, by DNA–DNA hybridization (zur Hausen et al. 1970).

The fanning out of the Nairobi material had many other interesting and some amusing byproducts. Blood and biopsies were sent to Philip Fialkow, human geneticist at the University of Seattle who was looking for unusual isozyme markers. One of his letters came with a big red label saying “top secret”! Not to be opened by anyone, but Professor Klein. Inside there was another envelope with a wax seal on it and the text “under no circumstances must this letter be opened by anyone but Professor Klein”. The letter said: Dear George, destroy this letter immediately when you have read it and make sure that its content is not communicated to Nairobi. You have sent us the blood of a Masai chief, living on the southern slope of the Kilimanjaro. On our request you have also supplied us with blood of his three wives and nine children. So far, we have tested eight of the children. None of them can be the descendants of the Masai chief. PS We just completed the ninth test. Paternity excluded.

Knowing a little about the Masai, I made a copy of the letter and sent it to the Svensson sisters, our Swedish secretaries in Nairobi, girls with a good sense of humor. They immediately wrote to Fialkow as follows: Dear Dr. Fialkow, the Masai are a highly ethical and moral people but their customs differ from ours. After the Masai chief has entertained a close friend of his age group for dinner, he walks around with the friend among the huts of his wives. He puts down his spear in front of one of the huts. That is where he invites his friend to spend the night. However, if anyone would try to get into a wife without being invited, he would be immediately killed. PS there is no need to keep this information confidential.

Following the confirmation that BL and NPC are the two most regularly EBV-carrying malignant tumors, their dominating position remained. The low differentiated or anaplastic form of NPC was found to carry latent EBV in nearly 100 %. This was independent of geography. It was equally true for the high-incidence Southern Chinese and the rare Western cases. Such a regular association must have a profound significance.

Today, several decades after the discovery of the association, the role of the virus for the etiology of the tumor is still unknown.

In BL, the situation is somewhat different. The virus is associated with about 90 % of the high endemic African tumors, but the worldwide sporadic BLs carry EBV in only 20–30 %. The rest contains no virus and has no traces of its genome.

An important missing link in the genesis of BL was provided by cytogenetic studies. A curious, unexpected convergence occurred at our laboratory. On the second floor, Francis Wiener, just about the best mouse tumor cytogeneticist of this time, worked on mouse plasmacytomas, together with Shinsuke Ohno from Japan. On the third floor, George Manolov and his wife Yanka Manolova, human cytogeneticists from Bulgaria, looked at the chromosomes of the incoming BL biopsies from Nairobi. The two cytogenetic teams did not have much communication. Frankly, they did not have much respect for one another. The mouse cytogeneticist did not think much about the work on human material that did not permit experimentation. For the human cytogeneticist, the mouse work appeared irrelevant.

And then suddenly, the two floors converged. Almost precisely, the same chromosomal translocations were discovered in mouse plasmacytoma and human BL. Subsequently, many other laboratories became involved in this work. The outcome was that the translocations act by juxtaposing the powerful *c-myc* oncogene to one of the three immunoglobulin loci. To my great surprise, the work of Phil Leder and Carlo Croce on BL and of Susan Cory and Ken Marcu on MPC confirmed my speculation that an eminent molecular biologist friend, Lennart Philipsson, called the most hair-raising extrapolation from the centimorgans to the kilobases, postulating that these translocations act by juxtaposing an oncogene to a highly active normal gene in that particular cell type (Klein 1981). It was the only hypothesis I ever made that turned out to be correct. The work was crowned by Michel Cole's demonstration that the oncogene was *c-myc* in both BL and MPC. I do not know of any other example in cancer biology where tumors of two different species, with entirely different pathogenetic histories, arise due to basically the same oncogene activation event in the same broad cell lineage as the only common denominator.

Thus, the *Ig/myc* translocation, rather than EBV, is the common feature of all BLs. But what is the role of EBV?

Prior to and in parallel with the cytogenetic developments, some monumental discoveries were made in the EBV field. They include the transforming and immortalizing activity of the virus for B cells, the role of the virally encoded growth transformation-associated proteins, and particularly EBNA2 and LMP1 for sustained B-cell proliferation and the causative role of the virus for mononucleosis. Moreover, the virus turned out to be the driving force for the immunoblastomas that arise from it genetically, iatrogenically (e.g., in transplant recipients) or by coinfection (HIV immunosuppressed patients). Importantly, EBV-carrying African BL cells do not express the full growth program. They do not need it because they are driven by *myc*.

In addition to BL and anaplastic NPC, other consistent tumor-EBV associations were discovered later. The relationship with Hodgkin's lymphoma (HL) is particularly noteworthy since there is both epidemiological and molecular evidence that the virus may be essential for the proliferative potential of the HLs that carry it.

Similar evidence is emerging for the EBV-carrying form of diffuse large B-cell lymphoma. The virus can also be carried by T-cell lymphomas. Its association with the T-cell-derived lethal midline granuloma is particularly consistent.

2 Is EBV a Cancer Virus?

Having completed the sequencing of the powerfully tumorigenic mouse polyoma virus, a considerable feat at the time, Fred Sanger felt that a human tumor virus should come next. On the advice of Beverly Griffin, he chose EBV, considered as the first human tumor virus. Paul Farrell carried out the job which had important consequences.

But is EBV really a tumor virus?

The immunoblastomas of the immunosuppressed are clearly driven by EBV as already mentioned. But are the immunoblastomas “truly malignant”? They tend to remain diploid and do not carry characteristic tumor-associated mutations. The frequently mutated p53 pathway remains wild type, as a rule.

3 What Is the Role of EBV in the Genesis of BL?

This question has no straightforward answer. But the presence of the virus in 90 % of the high endemic tumors cannot be a coincidence. I also attach considerable significance to the argument of Bill Sugden. He pointed out that the lack of synchrony between cell division and EBV episome duplication must lead to the loss of the viral genomes unless they are needed for the sustainability of the tumor. In fact, EBV-carrying BL lines can lose the virus in vitro, but a similar loss has never been observed in vivo. This indicates that some viral function may provide the in vivo tumor cell with a selective growth advantage.

4 The Lessons of HART Therapy

Malignant lymphomas have been a frequent complication of untreated AIDS. Part of them were EBV-driven immunoblastomas, but a substantial portion were Ig/myc-carrying BL or BL-like lymphomas. This picture changed radically after the introduction of HART therapy. In parallel with increase of the CD4+ T-cell count, the EBV-driven immunoblastomas practically disappeared. This makes sense in view of the highly efficient T-cell-mediated immune response against EBV-driven immunoblasts. The Ig–myc-carrying BL and BL-like lymphomas showed no similar tendency to decrease and are by now the most frequent lymphoma complication in HART-treated HIV carriers.

Ig-myc translocation-carrying cells with constitutively activated myc genes are prone to apoptosis. They need to be rescued by the cognate antigen or by B-cell stimulatory lymphokines and cytokines. HIV proteins linger around in the lymph nodes for a long time even under HART therapy. They may contribute to the survival of the Ig/myc translocation-carrying cells that are known to arise continuously as accidents of normal B-cell differentiation.

This argument is also pertinent to the question why chronic hyperendemic malaria and HIV are the only two conditions where translocation-carrying BLs arise in a relatively high frequency. A B-cell stimulatory environment is the characteristic for both conditions. Rescue from apoptosis by B-cell stimulatory cytokines may be the common responsible mechanism.

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