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Christian Münz *Editor*

Epstein Barr Virus Volume 1

One Herpes Virus: Many Diseases

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One Herpes Virus: Many Diseases

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Preface

We celebrated the 50th anniversary of the discovery of Epstein Barr virus (EBV) in Burkitt's lymphoma last year. During these 50 years of research on EBV, this first human candidate tumor virus has been found associated with many more malignant diseases in addition to Burkitt's lymphoma, including Hodgkin's lymphoma, nasopharyngeal carcinoma, a subset of gastric carcinomas, rare T/NK cell lymphomas, and many more. However, not only malignant diseases, but also some autoimmune diseases and the lymphocytosis of infectious mononucleosis have been found to be linked to EBV. In addition, we have learned to appreciate that continuous cell-mediated immune control prevents these EBV associated diseases, but cannot inhibit persistent infection, which the virus establishes in more than 90 % of the human adult population. Thus, EBV serves both as a paradigm for viral oncogenesis in humans and life-long immune control of chronic infection at the same time. The changes in the viral host cell and the host's immune control that determine the switch between these two states, continue to fascinate us and new experimental developments allow us to address this question in much more detail. Our ability to sequence EBV genomes faster and at lower cost allows us to explore the genetic diversity of EBV and its possible disease association for the first time. The recombinant EBV system allows us to generate mutant viruses to address the functional relevance of this diversity and new in vivo models of EBV infection, tumorigenesis, and immune control provide valuable insights into the pathologic relevance of the EBV characteristics that we have mapped during the last 50 years. With these tools in hand we should be able to unravel many more secrets that this human tumor virus keeps and develop vaccines against some of the EBV associated diseases in the next 50 years.

This exciting journey is summarized in the two book volumes in front of you. It starts with personal accounts of the discovery, tumor association, and immune control by pioneers of EBV research (Anthony Epstein, George Klein, Vivianna Lutzky, and Dennis Moss). It then continues with the knowledge on EBV genetics and epigenetics that has been gained (Paul Farrell, Paul Lieberman, Wolfgang Hammerschmidt, Regina Feederle, Olaf Klinke, Anton Kuthikin, Remy Poirey, Ming-Han Tsai, and Henri-Jacques Delecluse). An overview of EBV associated

diseases ranging from infectious mononucleosis and primary immune deficiencies to EBV associated tumors and autoimmune diseases completes the first volume (David Thorley-Lawson, Kristin Hogquist, Samantha Dunmire, Henri Balfour, Jeffrey Cohen, Ann Moormann, Rosemary Rochford, Paul Murray, Andrew Bell, Jane Healy, Sandeep Dave, Nancy Raab-Traub, Kassandra Munger, and Alberto Ascherio). In the second volume individual latent EBV gene products are then discussed (Lori Frappier, Bettina Kempkes, Paul Ling, Martin Allday, Quentin Bazot, Robert White, Arnd Kieser, Kai Sterz, Osman Cen, Richard Longnecker, Rebecca Skalsky, and Bryan Cullen). Viral entry and exit complete the virology chapters (Lindsey Hutt-Fletcher, Luidmila Chesnokova, Ru Jiang, Jessica McKenzie, and Ayman El-Guindy). The remainder of volume two is dedicated to the EBV specific immune response (Martin Rowe, Anna Lünemann, David Nadal, Jaap Middeldorp, Andrew Hislop, Graham Taylor, Maaike Ressing, Michiel van Gent, Anna M. Gram, Marjolein Hooykaas, Sytse Piersma, and Emmanuel Wiertz), in vivo models of EBV infection (Fred Wang, Janine Mühe, and Christian Münz), and EBV specific therapies (Stephen Gottschalk, Cliona Rooney, Corey Smith, Rajiv Khanna, Jennifer Kanakry, and Richard Ambinder). The resulting picture of 32 chapters on EBV biology will hopefully inspire many more young scientists to join research on this paradigmatic human tumor virus.

Indeed we might just have now the toolbox in hand not only to transfer discoveries in preclinical infection models to EBV, but also use EBV itself as a human model pathogen to learn more about the human immune system, viral dynamics in the human population, and the intricacies of EBV infection.

Zürich, Switzerland Christian Münz

Contents

Part III Viral Infection and Associated Diseases

Part I
History

Why and How Epstein-Barr Virus Was Discovered 50 Years Ago

Anthony Epstein

Abstract An account is given of the experiences and events which led to a search being undertaken for a causative virus in the recently described Burkitt's lymphoma and of the steps which ultimately culminated in the discovery of the new human herpesvirus which came to be known as Epstein-Barr virus (EBV).

Contents

Based on a lecture presented at EBV@50, the International Meeting in Oxford held in the week of 28 March 2014 to celebrate the 50th Anniversary of the first publication on the virus by Epstein, Achong and Barr on 28 March 1964.

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1 Introduction

The story I am recalling here arose from a sequence of linked chances which followed from one to the next in an extraordinary chain, with each coming at exactly the right moment for its significance to be recognized. As was memorably pointed out by Louis Pasteur 160 years ago, "Dans les champs de l'observation le hasard ne favorise que les esprits préparés" (*In the field of observation chance favours only the prepared mind*).

1.1 Early Chance Events Essential for Both "Why" and "How"

The chain started some 65 years ago when I began research at the Middlesex Hospital Medical School in London (founded 1836; since 1987, incorporated into University College London) and a quite unexpected death gave me access to one of the very earliest electron microscopes at a time when such things were exceptionally rare. Made in UK by Metropolitan-Vickers in Manchester in 1946 it was the first commercially available instrument of this kind, but is now an exhibit in the Manchester Museum of Science and Industry; sadly it was not persisted with and the subsequent market went to Holland, Germany, Japan and the USA.

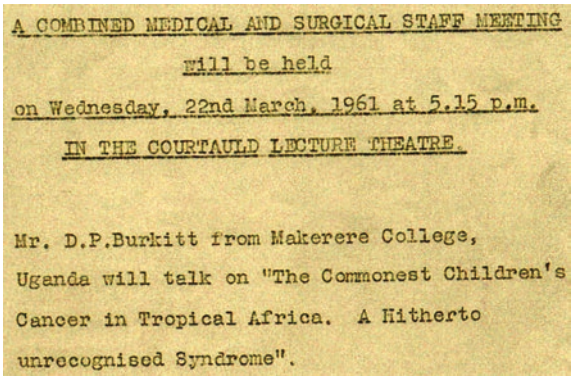
It was also a lucky chance that the Middlesex Hospital Medical School had an interest in the then deeply unfashionable chicken cancer viruses. So it was that I came to work on the Rous fowl sarcoma virus, the first virus known to cause malignant tumours. It was studied then by only a handful of people worldwide; indeed, so unfashionable at that time was the idea of viruses causing cancer in general that Peyton Rous (1879–1970) only got the Nobel Prize (1966) 55 years after he made his discovery (Rous 1911) because he lived to 86 by which time views on this subject had changed radically.

With the Rous virus I was able, using the electron microscope, to demonstrate its morphology and show for the first time that it was an RNA not a DNA virus (Epstein 1958; Epstein and Holt 1958). All this made me keenly aware of viruses which cause cancer and of the possibilities of electron microscopy.

1.2 A Subsequent Key Chance Leading to "Why"

This further chance was critically significant. In the 1950s a British Colonial Service medical officer based in Uganda came, when on leave in UK, to the Middlesex Hospital, London (Fig. 1), where he had a connection with the Academic Department of Surgery and his enthralling seminars were usually about

Fig. 1 The Middlesex Hospital, London, UK (founded 1745; 2005, replaced by the new University College London Hospital). Image courtesy of University College London Hospital NHS Trust archive


A COMBINED MEDICAL AND SURGICAL STAFF MEETING
will be held
on Wednesday, 22nd March, 1961 at 5.15 p.m.
IN THE COURTAULD LECTURE THEATRE.

Mr. D.P.Burkitt from Makerere College,
Uganda will talk on "The Commonest Children's
Cancer in Tropical Africa. A Hitherto
unrecognised Syndrome".

Fig. 2 Photograph of the notice of a talk by Denis Burkitt in 1961 at which he gave the first account outside Africa of the lymphoma which came to carry his name. The original notice is still extant

the exotic and extreme cases encountered in a developing country. Early in 1961 he came again, but this time he gave a very different kind of talk—the speaker was in fact Denis Burkitt (1911–1993), an unknown bush surgeon as he described himself, and his lecture (Fig. 2) was the first account he had ever given outside Africa of the lymphoma which brought him worldwide fame. Quite by chance I saw the notice of Burkitt's talk, and probably out of curiosity, I went.

After the first 20 min I was greatly excited by this strange malignant tumour of children in Africa affecting bizarre sites and fatal in a few months (reviewed in Burkitt 1963). But even stranger, Burkitt went on to present unprecedented preliminary data which showed that geographical distribution depended on temperature and rainfall. This suggested to me that a biological agent must play a part in causation and with my knowledge of tumour viruses I immediately postulated a climate-dependant arthropod vector spreading a cancer-causing virus. It turned out later that it was a cofactor which was arthropod borne (Burkitt 1969), but my idea focused correctly on the need to search for a viral cause.

Even as Burkitt was talking, I decided to stop my current work in order to seek for viruses in what became known as Burkitt's lymphoma—so excited was I that

after the talk I took the notice off a board (Fig. 2) and I have had it ever since. When Burkitt finished speaking, I was introduced to him, I invited him to my laboratory, and we agreed to collaborate. It was these quite unrelated chances which were responsible for "Why" the virus was discovered.

2 The Search for a Virus

So what about "How" the virus was discovered? That started with generous support from the then British Empire Cancer Campaign (founded 1923, became the Cancer Research Campaign 1970, became Cancer Research UK 2002) which funded me to visit Uganda a few weeks later. A first visit to Africa was quite daunting in the 1960s for unlike now, when even teenagers backpack widely, exotic travel was rare then. However, here too chance leant a hand because after World War II ended in Europe I had been posted to the Far East (Fig. 3) where the conflict continued with Japan, and having learned how things were done in the Indian Empire under the British Raj, it was easy to find my way around the British Ugandan Colonial Administration modelled on it.

Fig. 3 Inspection at Bareilly Cantonment in 1946 by Field Marshall Sir Claude Auchinleck, C-in-C India Command. Capt. M.A. Epstein (*right*)

The purpose of my visit to Uganda was, of course, to work out how a regular supply of lymphoma samples from Burkitt's patients in the capital Kampala could be flown overnight to my laboratory in London.

2.1 Reflections on Research Funding in the 1960s

Commenting on these events a much later Editorial aptly remarked "It is hard to imagine any current funding agency supporting a project based on the gut feeling of a young worker without any supporting data. Thank goodness that was not the case 40 years ago!"

2.2 The Beginning of "How"—Persistent Early Failures

For 2 years I applied the virus isolation techniques then in use to lymphoma samples with depressing negative results. Tumour material was inoculated into test cell cultures, embryonated hen eggs and newborn mice but without effects and direct examination in the electron microscope also proved fruitless. Failure to gain anything with this tool in relation to the lymphoma was especially disappointing in view of my early access to it. But additionally so since in 1956 I had gone, thanks to the Anna Fuller Fund of New Haven, to the Rockefeller Institute in New York (now the Rockefeller University) specifically to learn from George Palade (1912–2008; Nobel Prize 1974) at the time of his outstanding contributions to the earliest phases of biological electron microscopy and, indeed, to the very foundations of the whole of modern cell biology.

This long period without results was extremely alarming at a very insecure stage in my career. There was no employment law at that time—I had no letter of appointment, no terms and conditions, and no idea from year to year whether the Head of Department would feel inclined to reappoint me.

At this very low point I managed, unusually for a UK scientist then, to get a very small grant from the US National Cancer Institute. This $45,000 gave me some very modest independence and enabled me at the end of 1963 to recruit Dr Bert Achong (1928–1996) to help, once I had taught him, with the electron microscopy and Miss Yvonne Barr (as she then was) to assist with tissue culture of which she already had some experience. Before this I had worked for 15 years only with George Ball (Fig. 4), an absolutely reliable and completely unflappable young laboratory technician, who had provided indispensable support and continued to do so in the decades to come.

Fig. 4 George Ball in 1961, the absolutely reliable young laboratory technician who provided indispensable support before, throughout and long after the search for EBV. Image courtesy of Mr. G.R. Ball

2.3 An Idea Giving a Glimmer of Hope for "How"

In the event, an idea at this time proved more important than the grant. It occurred to me that if the tumour cells could be grown in culture away from host defences, a latent cancer virus might be activated and become apparent as I knew happened with certain chicken tumours (Bonar et al. 1960). However, doing this with a human lymphoma seemed unlikely since no type of human lymphocytic cell had ever been maintained in vitro for more than an hour or two (Woodliffe 1964). Nevertheless I tried repeatedly with the lymphoma using fragments in plasma clots, fragments floating on rafts and so on, but depressingly and quite predictably all failed.

2.4 Chance Provides the Key to "How"

Yet once again chance intervened in a big way. On Friday 5 December 1963 the overnight flight from Kampala was diverted to Manchester by fog and we were only able to retrieve our biopsy in the afternoon after the plane finally reached London. As usual the tissue was floating in transit fluid, but unusually this was cloudy. As it was getting late and the cloudiness was likely to be due to bacterial contamination, the feeling was that we could leave the laboratory for the weekend. But instead of discarding the specimen and going home I put a drop of the cloudy fluid on a slide and examined it with the light microscope as a wet preparation.

Rather than seeing the expected contaminating bacteria I was astonished to find that the cloudiness was due to large numbers of viable-looking free-floating tumour cells (Fig. 5) which had been shaken from the cut edges of the lymphoma sample during the flight.

This chance was in turn assisted by another, for I was immediately reminded that earlier that year on a visit to Yale Medical School I had learned that their Mouse Lymphoma Research Group had only succeeded in culturing mouse lymphoma cells by starting with suspensions of free-floating single cells (Fischer 1957, 1958) obtained in their case in ascitic fluid after growing the tumours in the abdominal cavities of mice.

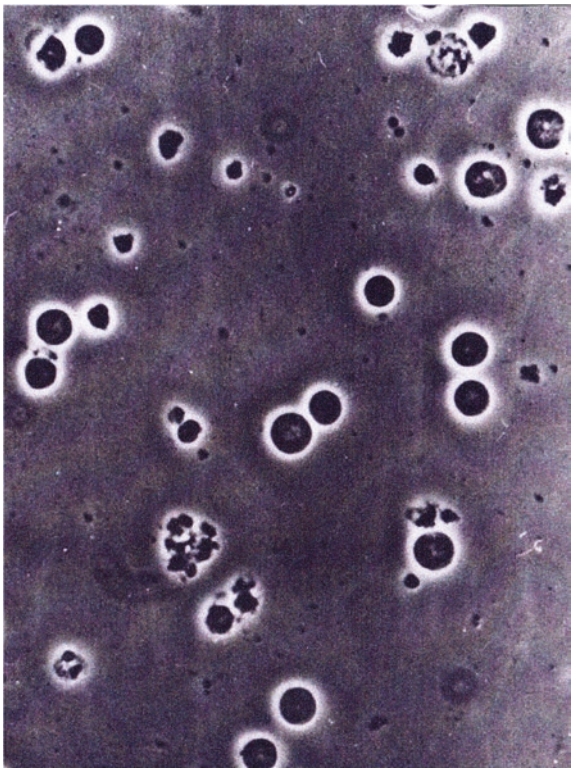


Fig. 5 Wet preparation of free-floating viable-looking lymphoma cells shaken from the cut edges of the lymphoma sample sent overnight from Uganda 4/5 December 1963. The appearance was reminiscent of a mouse ascites tumour. Phase-contrast light micrograph

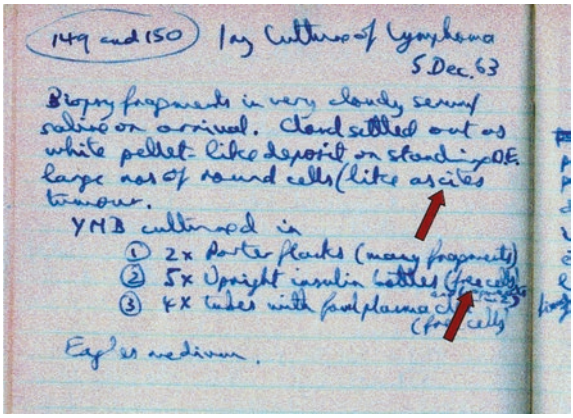


Fig. 6 Photograph of the author's laboratory notebook for 5 December 1963. Note the delayed lymphoma sample described as "like ascites tumour" (*arrow*) and set up for the first time in suspension culture—"free cells" (*arrow*)

Because of this, the free-floating cells in our delayed sample were described in my laboratory book (Fig. 6) as "like ascites tumour" and were set up for the first time in suspension culture (Fig. 6) as "free cells".

2.5 The End of the Beginning to "How"

Shortly after setting up the suspension culture a continuous lymphoma-derived cell line grew out (Fig. 7) which we labelled EB (Epstein and Barr) to distinguish its containers from the HELA, OMK, BHK and other banal cell lines we had in the laboratory, and suspension culture rapidly gave us more such lines from further Burkitt's lymphomas. It should be noted that 50 years ago there were no hoods and we worked on the open bench with rigorous aseptic technique in the updraft of a lighted Bunsen burner which carried atmospheric contaminants away. Very early on we used extremely small conical flasks which allowed more culture fluid without increasing the depth than with straight-sided containers, since depth critically affected the diffused oxygen tension around the cells resting on the bottom, and this system had the advantage that it could readily be scaled up as the cells became plentiful.

This was the first time that human lymphocytic cells had ever been grown long term in vitro, and when the account of the successful procedure was sent for publication, a leading journal's expert referees were unwilling to believe that human lymphocytic cells could be cultured at all. Yet suspension is now the standard technique to grow such cells used worldwide today for a huge number of different types of research.

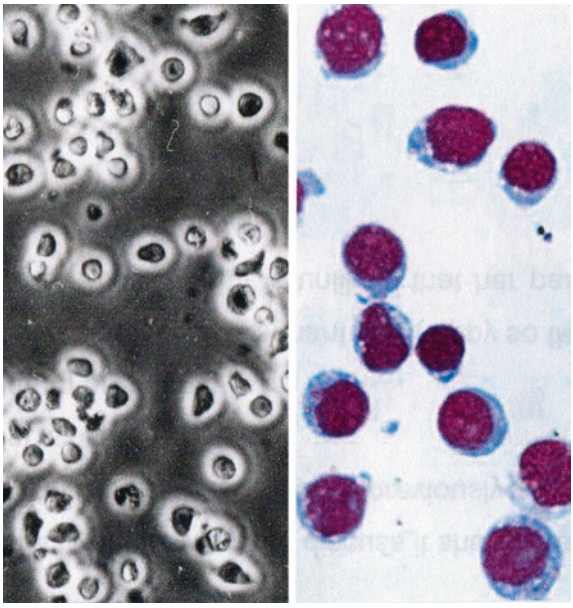


Fig. 7 Light micrographs of the first ever culture of Burkitt's lymphoma-derived lymphoblasts designated EB1. Phase contrast of live cells (*left*) and Giemsa-stained fixed cells (*right*)

3 The Final Breakthrough to "How"

All efforts to show a virus in EB cells using standard contemporary biological tests failed, so as soon as material could be spared, some cells were fixed, pelleted and embedded for electron microscopy. But it should be emphasized that this was not accepted then as a method for demonstrating viruses; dogma required that they should be shown by their biological activity or by finding the antibodies they induced. It was not credited that they could be recognized morphologically. Indeed, at this time when electron microscopy was rare and little understood, the images obtained of biological material were considered by many as artefacts of fixation and processing.

It is worth mentioning here that a notable exception to such views was provided by Oxford's Professor Sir Howard Florey (1898–1968; penicillin Nobel laureate 1945, later Lord Florey of Adelaide and Marston); not one to miss a new and important advance he had come himself to my very small laboratory in London on 21 January 1959 to see what electron microscopy was about in preparation for setting up a unit in his department.

3.1 "How" the Virus Was Found

As regards images of viruses, my time with George Palade had convinced me that they could be recognized, and classified at least into families, by their appearance as had been done for bacteria with the light microscope for 100 years.

I examined the first EB cell preparation with the electron microscope on 24 February 1964 and was exhilarated to see unequivocal virus particles in a cultured lymphoma cell in the very first grid square I searched. I was extremely agitated in case the specimen might burn up in the electron beam—I switched off, I walked round the block in the snow without a coat, and when somewhat calmer I returned to record what I had seen.

I recognized at once that I was looking at a typical member of the herpesvirus group (Fig. 8) with which I was already very familiar and noted it as such in my electron microscope laboratory book (Fig. 9) "virus, like herpes", but there was no means of knowing which herpesvirus it might be. However, it did seem quite extraordinary that a herpesvirus was producing virus particles in a cell line yet was so biologically inert that it did not destroy the whole culture as the known herpesviruses would have. Accordingly I rapidly set about reporting the discovery with my new assistants Bert Achong and Yvonne Barr (Fig. 10). The resulting paper (Epstein et al. 1964) appeared on 28 March 1964 and became a Citation Classic in 1979, and the 50th anniversary of its publication was celebrated at an International Meeting in the week of 28 March 2014.

The unusual inertness was reinforced when biological tests for herpesviruses were applied to the EB cells and all proved negative. At this point I became

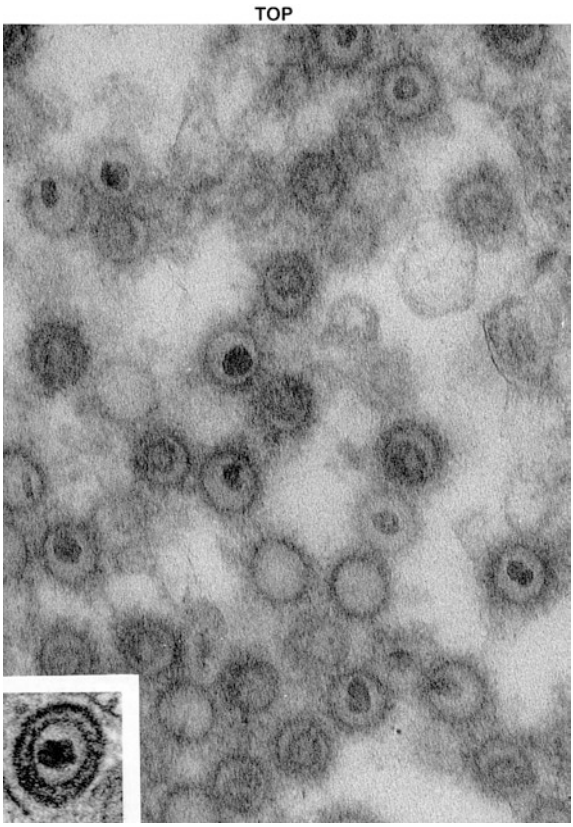


Fig. 8 The first electron micrographs of EBV. Immature virions assembling in the cytoplasm of a cultured EB1 lymphoma cell; *inset*, a mature enveloped particle. These images were recognized at once as a herpesvirus

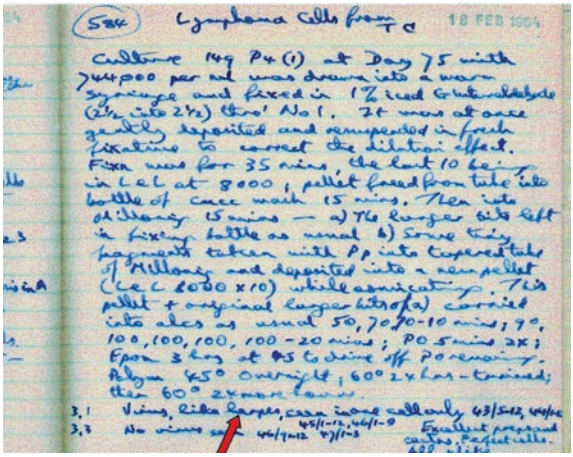


Fig. 9 Photograph of the author's electron microscopy notebook dealing with the EB1 cells harvested on 18 February 1964 and examined, after the usual delays for processing, on 24 February 1964. Note entry "virus, like herpes" (*arrow*)

Fig. 10 M.A. Epstein, B.G. Achong (1928–1996) and Y.M. Barr in 1964 at the time of the first publication on EBV (Epstein et al. 1964)

concerned that something unnoticed in our procedures was inactivating the virus and it was clearly urgent to have the tests repeated in some other laboratory.

I approached two leading British herpes virologists, but neither was interested in our unorthodox findings, and so it came about that I contacted my friends the husband and wife virologists Werner and Gertrude Henle (1910–1987; 1912–2006) at the Children's Hospital in Philadelphia.

EB cells were flown from my laboratory to Philadelphia, the Henles rapidly confirmed the biological inertness of the virus, and we then reported jointly that it was a new member of the herpes family (Epstein et al. 1965).

3.2 Naming the Virus

Following my sending the virus to the Henles, they soon subsequently referred to it as "EBV" (Henle et al. 1968) after the EB cells in which it had come to them, and this name caught on and was rapidly universally adopted.

3.3 Characterization of the Uniqueness of the Virus

In addition to the biological inertness of the virus, its immunological singularity was soon demonstrated in Philadelphia (Henle and Henle 1966) and in my laboratory using quite different techniques (Epstein and Achong 1967). Shortly after this its novel biochemical nature was also established (zur Hausen et al. 1970), and 14 years later the complete viral genome was sequenced (Baer et al. 1984).

In the light of subsequent knowledge of the very limited range of cells with receptors for the virus, the failure to show biological activity is readily understandable, but it was very puzzling at the time. It was indeed fortunate that work on the lymphoma cells and the search for a virus was undertaken in a laboratory where a rare electron microscope was in daily use (yet another chance) as otherwise the extreme inertness could have left it undiscovered.

Table 1 Epstein-Barr virus—research publications (from PubMed)

28 March 1964–28 March 2014	30,995
1984	525
2004	1079
2013	25/week

4 Concluding Remarks

EBV was in fact the first virus to be found solely by electron microscopy, and the story of its discovery thus acted out a little joke published over 100 years ago before viruses were known or electron microscopes dreamt of:

> The microbe is so very small
> You cannot make him out at all
> But many sanguine people hope
> To see him through the microscope
>
> (Belloc 1897)

But the huge extent of work on EBV following its finding by electron microscopy is not generally realized even by experts in the field and is therefore worth a comment. In the first 50 years since the discovery there were more than 30,000 peer-reviewed publications on EBV (Table 1). Of course in the early years the numbers were very small, but as the decades went by they increased dramatically (Table 1, cf 1984 and 2004) and finally in 2013 they were running at 25 per week. It is an arresting thought that each author of a chapter in the present book is making a contribution, however small, to the vast worldwide undertaking of accumulated EBV research.

The reason for the wide interest in EBV has been, of course, because it was the first putative and then the first definitive human cancer virus. Interestingly, in the 50 years that the virus has been known to science human tumour virology has moved from the distant margins of the biomedical agenda to the very centre and in recent years to the very top with the introduction of anti-tumour virus vaccine programmes to prevent significant human cancers.

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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.

Contents

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 clockwork 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