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Recent Studies with EB Virus[1]

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It is now 11 years since the discovery of EB virus (1), and during that time an immense amount of information has been accumulated on this new human herpes virus by large numbers of investigators in many different countries (for reviews, see 2–4). With so many studies currently going forward on this agent and so many new workers coming into the field, it was thought that a brief historical survey on the early findings with EB virus might be timely, coupled with a consideration of some still outstanding problems connected with its biological behavior in man.

DISCOVERY OF EB VIRUS

On March 22, 1961, D. P. Burkitt addressed a combined medical and surgical staff meeting at the Middlesex Hospital Medical School, London, and presented for the first time outside Africa an account of epidemiological studies suggesting that the distribution of the highly unusual tumor that now bears his name was connected with geographical features affecting climate. From this it was immediately evident that some biological factor must almost certainly be involved in the etiology of the tumor, and the exciting possibility arose that this might be an infectious agent spread, perhaps, by a climate-dependent arthropod vector. It was therefore decided to undertake extensive investigations of the Burkitt lymphoma (BL), and biopsy samples from such tumors were flown from eastern Africa to London and tested in various ways for the presence of viruses. Inoculations were made into embryonated hen eggs, test tissue culture systems, and newborn mice, but these early experiments proved uniformly negative. Thin sections of tumor samples were therefore searched in the electron microscope in an effort to find unusual viruses that might not be demonstrable by standard isolation procedures, but direct examination of tumor samples likewise proved negative. It was then considered (5) that success might be achieved if Burkitt lymphoma cells could be grown *in vitro* away from host defenses so that an oncogenic virus might be able to replicate, as happens with cultured cells from certain virus-induced animal tumors whose causative agent may not be readily detectable *in vivo* (6). This concept provided the rationale for the attempted long-term cultivation of Burkitt lymphoma cells and led directly to the discovery of EB virus. At the time, however, the prospects for establishing lines of lymphoblastoid cells from Burkitt tumors were indeed unpromising since no member of the human lymphoblastic series of cells had up till then been grown continuously *in vitro* despite innumerable efforts ever since the earliest phases of the tissue culture technique (7).

However, success was achieved toward the end of 1963 (8); when samples from the continuously growing Burkitt tumor cells were examined with the electron microscope, virus particles were observed in a cell within the first grid square to be searched, and the virus was immediately recognizable morphologically as a typical

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member of the herpes group (1). Although when first seen there was no way of knowing which herpes virus was present, it soon became clear that the agent was highly peculiar since it proved biologically inert in the usual test systems (9), and evidence for its immunological uniqueness was soon forthcoming. Antisera to known herpes viruses did not react in indirect immunofluorescence tests with cells carrying the virus (10 and 11), while artifical antisera produced in rabbits by injecting purified EB virus showed that the herpes agents in several different Burkitt tumor cell lines were immunologically identical and quite distinct from known human herpes viruses (12 and 13).

New workers in EB virology are sometimes curious as to how the agent first got its present name. In the early stages considerable help with the establishment of continuous cultures of Burkitt lymphoblasts was provided by Yvonne Barr, who was working for a Ph.D. in the laboratory. When the cells eventually grew, they came to be labeled by the laboratory shorthand of "EB" cells (Epstein/Barr—to distinguish them from other cells being cultured at the time), and when sent elsewhere as a source of the virus, the recipients in other laboratories came to designate the virus itself as EB virus. Shortly after this, Dr. Barr married and exchanged a productive career in science for a productive career in maternity.

EB VIRUS AS A CANDIDATE HUMAN TUMOR VIRUS

Although EB virus is of general interest as a new herpes virus of man, its main significance relates to its remarkable association with two human cancers, African BL and nasopharyngeal carcinoma (NPC). In connection with this association, the following points have been firmly established and repeatedly confirmed:

1. Authenticated cases of African BL and NPC only occur in individuals infected by the virus; seroepidemiology has shown that patients with these two tumors have antibodies to the virus, many in high titer, whereas among controls and patients with other malignancies, seronegatives are found.

2. The viral DNA is present in all the malignant tumor cells and determines the expression in them of virus coded neoantigens, very much as happens with tumors known to be caused by animal oncogenic DNA viruses; not surprisingly, at least with BL, virus production is activated in some of the tumor cells when they are placed in culture.

3. EB virus is a powerful stimulator of lymphoproliferation *in vitro*—it confers the property of continuous growth on human B lymphocytes together with any changes reminiscent of malignant transformation. Normal human peripheral lymphoid cells free of EB virus genome will not grow in tissue culture unless the virus is added. In contrast, peripheral lymphoid cells from seropositive individuals will grow continuously in culture since a certain proportion of them already harbor the virus DNA.

4. The virus is a powerful stimulator of lymphoproliferation *in vivo* as the cause of Paul–Bunnell positive infectious mononucleosis.

5. The virus is carcinogenic experimentally *in vivo,* causing malignant lymphoma when inoculated in South American subhuman primates, in such a manner that it has been possible to fulfill Koch's postulates with the system.

6. Animal herpesviruses behaving similarly to EB virus are now known to cause malignant lymphoma or carcinoma in natural or experimental hosts. These points have all been discussed and fully documented elsewhere (2–4, 14, 15).

Since EB virus occurs as an ubiquitous infection in human populations while the

tumors with which it is associated have a geographically limited high incidence, other factors besides EB virus (assuming it actually is carcinogenic) must be involved in causation. It has long been recognized that high incidence areas of BL are determined by temperature and rainfall (16, 17) and any cofactor would therefore appear to be environmental; holoendemic malaria has been suggested in this connection (18), and there are cogent reasons for believing that it may provide a suitable cellular response on which EB virus may then act as a carcinogen. With regard to NPC, here it has been recognized for some time that genetic cofactors are of special importance; the disease has a very high incidence only in populations of southern Chinese origin (19), and among these, certain genetically determined individuals are unduly at risk (19, 20). However, this cannot be the whole story since the children of immigrant southern Chinese living in California have a lower incidence of NPC than their parents (19).

EB VIRUS INFECTION IN NORMAL INDIVIDUALS

Although the circumstantial evidence incriminating EB virus as an element in the etiology of BL and NPC is weighty and impressive, many problems remain. A great deal is known about the behavior of this virus in human populations in which, as already mentioned, it causes widespread infections (see 21 for review). In contrast, relatively little is known of the biological behavior of EB virus in the infected person, and this has led to the recognition of certain seeming paradoxes.

It has long been known that continuous lymphoblastoid cultures can be readily established from biopsy samples of BL and both the lymphoma cells and the cells of the tumor-derived lines contain EB virus DNA (22 and 23). It is also known from studies of cytogenetic, IgM specificity, and isoenzyme markers that when lymphoid lines are obtained from these lymphomas, it is the tumor cells, already malignantly transformed *in vivo,* which usually grow out to form the cultures (24–27). On the other hand, EB virus DNA-containing continuous lymphoblastoid cultures can also be grown from the peripheral lymphocytes of healthy individuals and patients with infectious mononucleosis provided that the donors are seropositive (2–4), and this raises the important question as to the state of the virus *in vivo* in the few genome-containing circulating lymphocytes from which these nontumor-derived lines originate. If EB virus really is an etiological agent in the malignant transformation underlying BL, can it be present in the tumor cells in the same state as that in which it infects the peripheral lymphoid cells in the huge number of seropositive people who do not have lymphomas? In order to elucidate this question, investigations have recently been undertaken to look at the state of the EB virus genome in the peripheral lymphoid cells of one group of tumor-free individuals, namely, patients with IM; the experiments have used cocultures of peripheral lymphoid cells from IM patients with fetal cord blood lymphoid cells of opposite sex, grown together in microtest plates by methods which have already been described (28).

In a first set of experiments, equal numbers of IM leukocytes and fetal leukocytes of opposite sex were cocultured and observed for the onset of transformation. The first cell clumps seen were subcultured, and samples of the resulting cell lines were taken for chromosome sexing 24 hr after this and after a second subculture, 6–8 days later. Control cultures were set up with IM leukocytes or fetal leukocytes alone. In these coculture experiments, transformation occurred regularly, and the resulting transformed cells were made up of mixtures of IM-derived and fetal-derived cells with a very marked tendency for fetal cells to predominate.

Transformation did not occur when fetal cells were grown alone, while IM cells alone gave transformed lines in almost every case. This result suggested that IM cell lines do not arise *in vitro* by the direct outgrowth of EB virus genome-containing cells already transformed in the patient such as occurs with cell lines from biopsies of Burkitt lymphomas. Instead, something appears to be liberated from IM cells in culture that brings about the *in vitro* transformation of neighboring virus-free cells from which the lines then derive; the many fetal cells that gave lines in the cocultures must have been transformed in this way. In a second group of experiments, the incidence of transformation was investigated in IM cultures, in fetal cultures from donors of opposite sex, and in cocultures of X-irradiated IM leukocytes with the paired fetal cells. The origin of the transformed cells that arose in these cocultures was determined as before by chromosome sexing. Comparison of the incidence of transformation in the cultures of IM leukocytes alone with the incidence where IM leukocytes of similar origin were X-irradiated and grown with fetal leukocytes showed good correlation, and it was evident that IM patients whose lymphocytes transformed readily gave a high incidence of transformation when their lymphocytes were X-irradiated and cocultured with fetal lymphocytes, and that it was only the latter that were transformed in such cocultures. This close correlation between the ability of IM cells from a given donor to transform on their own and their ability following X-irradiation to transform cocultivated fetal lymphocytes suggests a similar transformation mechanism in both situations.

Evidence that this transformation mechanism actually depends on the production of infectious EB virus particles was provided by further experiments in which the ability of different samples of IM leukocytes to transform when cultured alone was found to correlate well with the transforming ability extractable from the samples, since the extracts could be neutralised by antiserum to EB virus.

This conclusion was studied further in experiments in which the transformation of IM leucocytes was followed in cultures containing either antiserum to EB virus or negative serum. The results obtained gave evidence of a different kind, which confirmed the role of infectious EB virus particles in the emergence of lines of transformed cells from IM lymphocytes; for where IM cells were cultured at rather low concentrations in the antiserum, complete abolition of transformation occurred, as compared to cultures in which normal serum was used. This abolition was shown to be specifically antiviral, not the result of some cytoxic effect of the antiserum on transformed cells, since fetal lymphocytes exposed to EB virus and then immediately cultured in the antiserum showed no cytotoxic effects, and the usual transformation seen with similarly treated fetal cells grown in negative serum took place. These investigations have been described in detail elsewhere (28).

DISCUSSION

The evidence obtained in this work from experiments of several different kinds clearly shows that continuous cell lines do not arise *in vitro* from peripheral lymphoid cells of patients with IM by the outgrowth of cells already transformed *in vivo*, such as occurs with the lymphoid lines of Burkitt tumor origin. In contrast, the present results demonstrate that lines arise from IM lymphoid cells by a two-step mechanism. First, the EB virus genome carried by a small number of peripheral lymphoid cells is activated to a productive infection with liberation of infectious virus particles, when the cells are placed in culture. Second, the virus particles infect nearby normal lymphocytes which then undergo *in vitro* transformation to give

lines. In the sparse cultures with little possibility of cell to cell contact, this second step was completely abolished by antiserum to EB virus. This mechanism is not thought to be peculiar to the origin of IM-derived lines but is considered to be the usual process whereby lines arise *in vitro* from the lymphoid cells of any seropositive tumor-free individual. Such people harbor EB virus for life and regularly have a small number of peripheral lymphoid cells carrying the viral genome, and it would appear that such cells are merely more numerous during primary infections accompanied by the disease manifestations of IM (29).

The results presented clearly imply that EB virus DNA is present in the peripheral lymphoid cells of IM patients as a different type of infection from that shown by the virus in the malignantly transformed cells of Burkitt lymphomas; the latter, malignantly transformed *in vivo,* grow directly to give tissue culture lines (24–27), whereas IM lymphocytes depend on virus production to do this *in vitro* where the growth of cell lines was inhibited by nontoxic antiserum to EB virus. In this connection, parallel cytogenetic studies on the cells of lymphoblastoid lines of malignant and nonmalignant origin have demonstrated specific differences in chromosome banding between the two groups (30).

An understanding of the various forms of infection possible with herpes viruses as a family readily explains these two different kinds of infection with EB virus in human lymphoid cells. It has been pointed out elsewhere (21) that besides the straightforward productive infections caused by herpes viruses with virus replication leading always to cell death, viruses of this family also exhibit two kinds of nonproductive infection. In one, the virus DNA is unexpressed for long periods but can be activated to virion production, as happens with herpes simplex virus and varicella-zoster virus; EB virus in the lymphoid cells of seropositive individuals seems to follow this pattern. In the other type of nonproductive infection, the viral genome is expressed to cause malignant transformation with the accompanying production of viral determined neoantigens, as is seen in the animal lymphomas caused by herpes virus saimiri (31) and the herpes virus of Marek's disease (32, 33).

In the context of our general knowledge of the relationship of EB virus to African BL and NPC, the results of the experiments discussed here have removed an apparent difficulty in accepting that EB virus might be carcinogenic because they indicate that there seem to be two separate mechanisms for establishing human EB virus-containing lymphoid cell lines in culture, one relating to tumor cells and the other to viral genome-containing cells from individuals free of malignant disease. And this in turn suggests that EB virus causes nonproductive infections without expression of the viral genome in nonmalignant cells, in contrast to the nonproductive infections with expression of the genome that appear to occur in the malignant cells of African BL and NPC.

If these distinctions are borne in mind, difficulties in accepting EB virus as an oncogenic agent become apparent rather than real, and the behavior of EB virus in its various forms of infection is seen to conform exactly to the patterns followed by other members of the herpes virus family.

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