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Immortalization of Human Lymphocytes by Epstein-Barr Virus

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Epstein-Barr virus (EBV) confers upon normal lymphocytes derived from bone marrow the ability to proliferate indefinitely in a test tube. This process, called immortalization, is crucial to the pathogenesis of EBV infections. Inside the immortalized lymphocyte the EBV genome exists as a complete multicopy circular plasmid which is probably not integrated into the cell chromosome. Most of the viral genetic information is not expressed. However, at least six to eight separate regions of the EBV genome encode viral products which are made in the immortalized cell. The identification of the function of these few genes holds some interesting answers to questions concerning the biochemical mechanisms of control of lymphocyte growth and differentiation.

A crucial point of interaction between a parasite and its host underlies the pathogenesis of most infectious diseases. This interface is often called a site of "tropism," a place on or in a specialized target cell where effects occur which are responsible for the pathologic and ultimately the clinical signs of the disease. Examples come readily to mind. There are respiratory viruses with characteristic affinity for upper airways (common cold viruses), middle airway (croup associated viruses), and lower airways (respiratory syncytial virus). Although they initiate infection in the gut, and traverse the blood, polioviruses ultimately home to anterior horn cells of spinal cord [1]. Rabies virus travels to the temporal lobe via peripheral nerves. Although some other viruses are not nearly so finicky in their preferences, even those which affect many tissues, measles, for example, can be tracked to a favorite haunt, such as a small patch of buccal mucosa near the parotid salivary gland duct.

Virologists seldom enjoy the opportunity to study these fine-tuned viral adaptations to specialized target cells. Until recently it has been difficult to grow well-differentiated cells in tissue culture so that they retain their unique characteristics. We have a vague feeling that many of these tropisms are due to receptors on the outside of cells for specialized portions on the outside of viruses, but we do not know much about intracellular events which govern the interactions of viruses within specialized cells. However, in at least one instance we can now regularly put a differentiated cell in culture and study intracellular events which underlie the pathogenesis of an infection.

In the 1960s techniques of tissue culture had advanced to the point where it became possible to grow continuous cultures of certain types of human lymphoid

cells [2]. Often these cell lines were derived from patients with various leukemias and lymphomas, but occasionally they were established from normal individuals or from patients with diverse diseases. In the mid-1960s it was found that some, but not all, of these lymphoid cell lines contained a new human herpes virus, Epstein-Barr virus (EBV), which was first seen in a lymphoid line from a Burkitt lymphoma [3]. Could this new virus be responsible for the continuous growth of lymphoid cell lines?

The first experiment which suggested that this might be the case was reported in 1967 by the Henles and their associates [4]. They lethally X-irradiated an EBV carrier Burkitt lymphoma line derived from an African boy called Jijoye, and co-cultivated the X-irradiated cells with leukocytes from a female infant. After a number of weeks a lymphoid line was established which had a female karyotype. Although the infant's cells before co-cultivation contained no identifiable viral antigens, the established line contained an antigen which was also found in the Burkitt lymphoma cells. This type of experiment was carried out in other laboratories at about the same time, using X-irradiated EBV carrier cells established from patients with leukemia. Subsequently it was shown that partially purified EBV rather than cell-associated virus was capable of inducing the continuous growth of the lymphocytes [5]. The proof that EBV was responsible was based ultimately on the demonstration that growth of the normal cells could be inhibited if antibody to EBV was included in the system [6].

TRANSFORMATION VERSUS IMMORTALIZATION

Since EBV was found first in lymphoma cells, the question naturally arose whether acquisition of the ability to grow continuously in a test tube was tantamount to a change from a normal cell to a cancer cell. This question has not been easy to answer. Most of the evidence suggests that, while enhanced *in vitro* growth is often a property of a cancer cell, it is not the whole story. Thus soon after the phenomenon was put on a firm experimental basis, there arose the important question of what to call it. The word transformation is generally used to describe such events, but transformation also describes many unrelated phenomena including the uptake of DNA and expression of new information by bacteria (Avery's transformation), the response of lymphocytes to mitogenic stimuli, such as phytohemagglutination, and oncogenic transformation by tumor viruses. Since the question of tumorigenicity of the growing lymphocytes was unresolved, I tried to find another name, and hit on immortalization [7]. This term has drawn criticism as being presumptuous, for we shall never know whether a lymphocyte is immortal, but it has withstood the test of usage. In fact, as we become more aware of the sequence of changes which accompany the conversion from normal to malignant, its usage has increased.

TARGET CELLS

All lymphocytes which have been immortalized *in vitro* by EBV show attributes of B cells; usually they synthesize immunoglobulin. It is still unknown whether immature B-cell precursors can be immortalized. The most susceptible cell in laboratory studies seems to be a resting, well-differentiated, B cell [8].

This matter of target cell is of more than theoretic interest; the nature of the target cell is likely to influence the features of the disease. If it could be shown that EBV immortalizes a stem cell and there were unambiguous ways to identify such cells, then it might be possible to learn whether some B-cell malignancies associated with EBV were due to immortalization of B stem cells, an unusual target.

The target cell ultimately influences the expression of the EBV which immortalized it. All lymphocytes tend to inhibit the expression of EBV, but some more than others. If the lymphocytes come from umbilical cord blood, they tightly restrict EBV expression; from adult blood they are more relaxed; the lymphocytes of some New World primates permit considerable expression of EBV in the transformed cells, thus providing a source of virus for laboratory study [9].

These host restrictions on EBV expression in the immortalized cell may also influence the pathogenesis of the EBV-associated diseases. Clinical expression of EBV infection in the young child is generally less intense than in the older child. Since we know now that the pathogenic features of mononucleosis are principally due to the immune response, it is not too great a leap to posit that the subdued immune response in the young child is somehow related to the muted behavior of the virus in young cells.

EVENTS LEADING TO IMMORTALIZATION

It is possible to study the events leading to immortalization in cell culture. Therefore it should eventually be possible to recreate the cellular and molecular pathogenesis of the single most important aspect of EBV infections. These events are now discerned only in rough outline. There is attachment to a receptor on the B cell, thought to be the receptor for C_3 or something closely linked to it. Adsorption and penetration are quickly accomplished, but the details of how the virus enters are not known. The first recognizable event, after six to eight hours, is the synthesis of a nuclear neo-antigen called EBNA; about 20 hours after infection, cellular DNA synthesis is stimulated and about 16 hours later the first EBNA-positive cells are seen to enter mitosis [10]. There may be some further events which are needed to solidify the immortalization reaction, which does not begin in earnest until about five days after infection, when cell numbers begin to increase.

At some point during immortalization the EBV genome reproduces itself, not massively up to several thousand copies per cell as occurs in a productive viral infection, but cautiously and conservatively—usually somewhere between five and 50 copies per cell. After immortalization, this copy number is usually fairly constant [11]. "Genome amplification" is a regular occurrence and one cannot help feeling it is in some way tied in a causative way to immortalization.

IMMORTALIZED CELLS CONTAIN A LARGE PORTION OF EBV DNA OR ALL OF IT

The EBV genome probably contains at least 50 to 100 genes, based on its size alone. Most of these genes are likely to function in the replication of mature virus and, as such, are probably not involved in the immortalization process. Yet the immortalized cell invariably contains a nearly complete copy of the genome. Since no virions are being made in the majority of cells, most of the genes must be shut off. Nonetheless the whole viral DNA is present if one looks for it by biochemical means (Southern blotting) or by biologic tests. It is possible to recover infectious virus from immortal B cells by X-irradiating them and co-cultivating them with fresh lymphocytes [9].

Thus, lymphocytes immortalized by EBV are excellent cells in which to analyze the biochemical basis of viral latency. They contain a masked viral genome which is periodically spontaneously activated in some cells and which can be induced to replicate by certain chemical and physical stimuli.

It takes energy to conserve many copies of this large viral genome in a lym-

phocyte. There must be a reason for maintaining this entire genome, even though new virus is not made. Several explanations have some basis in experimental observation. Inside the cell the genome is circular; in the virion the genome is linear [12]. Circularization probably requires repeated sequences at the ends of the molecule. If the molecule is interrupted circularization may not occur, unless the ends are preserved. Inside the immortalized cell the genome is amplified; this may take place as the result of viral enzymes involved in DNA synthesis. In fact, the synchronous replication of the Epstein-Barr viral plasmid with its host cell and its partition to daughter cells may be under the control of viral genes.

Although many viral genes are shut off in the immortalized cells, a considerable number of areas of the genome are expressed as RNAs. There are at least six to eight transcribed regions scattered over the genome [13]. This may be the real reason why we see the whole viral genome in the cells. We know little of the business of these active regions; one region encodes two small RNAs which do not function as messenger RNAs, but become complexed with protein and serve some as yet unidentified function [14]. Another transcribed region encodes the Epstein-Barr nuclear antigen, as yet still mysterious in terms of structure or function.

HOW MIGHT THE IMMORTALIZING GENES WORK?

We are beginning to know something about how RNA tumor viruses transform cells or cause tumors, but the mechanism of action of the transforming genes of DNA tumor viruses is still mysterious. The two major pathways to tumorigenesis by retroviruses are to code for new proteins which are protein kinases or to integrate into cellular DNA and to cause cellular genes to be expressed because of promoters or because of gene rearrangements [15]. The product of these cellular genes may also function as protein kinases.

It is perhaps a naive way of looking at it, but since EBV is a large complex virus, it seems likely that the immortalization reaction too is going to be complex. Perhaps those biologists are wise who study the simplest systems. At any rate, since many viral genes are expressed in immortalized cells, I would predict that the products of these genes interact in some way to produce a cascade of effects. Probably most of these genes function primarily to maintain the vegetative state of the viral genome; that is, they serve to replicate and conserve the copy number. Perhaps the immortalization reaction is entirely a by-product of the viral genome's efforts to preserve itself. On the other hand, the virus may need to immortalize the cell first in order for the cell to supply some essential function which the virus requires and which cannot be found in a resting lymphocyte.

One attractive hypothesis is that the virus either encodes or induces a "hormone" or a receptor for a hormone in the culture medium. This hormone may be a perpetual mitogen.

The immortalization reaction is well on its way within 20 hours after infection when the cell is pushed into DNA synthesis. This is fast transformation and bespeaks the existence of viral gene products which act directly, rather than by some indirect mechanism such as integration and promotion of expression of cellular genes. In fact, there is little evidence that EBV integrates into cellular DNA.

The truth is we do not really know how this process might work. We badly need mutants which are deficient in immortalization. Only one such mutant exists, probably as the result of an event which occurred by chance while the virus was being propagated in the laboratory. We know now that, by comparison with its parent,

this mutant has a single deleted segment of viral DNA [16]. No doubt this missing piece of DNA performs a function which is essential in initiating immortalization. Aided by the powerful new methods of recombinant DNA and gene transfer, we should be able to learn what these missing genes do.

IMMORTALIZATION IS NOT NECESSARILY ONCOGENESIS

A cell immortalized in a test tube or in a patient does not often become a cancer, though it has the potential for becoming a malignant cell. Some nonhuman primates and certain immunodeficient patients lack one or more components of the immune system which are involved in detecting and eliminating immortalized cells. This is an example of a host-determined susceptibility to malignancy.

The lymphomas which are the result of the unbridled growth of immortalized cells in immunocompromised hosts are polyclonal; they derive from proliferation of a number of different cell clones which have probably received the virus in separate events [17]. Some would prefer not to call these true neoplasms, but these seem to me to be semantic distinctions. The immortalized cell in an immunodeficient host shows all the antisocial properties which are attributes of cancer cells—unlimited growth and invasion of normal boundaries between tissues and organs.

Unanswered is the question whether immortalization by EBV can also set the stage for the type of malignancy which occurs in immunologically normal persons. In the case of Burkitt lymphoma, it is likely that there are events which occur subsequent to immortalization. These probably include the chromosome abnormalities which are characteristic of the disease, and may well involve other somatic cell mutations which we can not yet identify. The idea of tumor progression has been on the minds of biologists who study cancer at least since Peyton Rous. Even after we have a clearer idea of what viral genes lead to immortalization, we shall be left with questions about the progression to malignancy. Does the virus participate in this process, too, in a direct way? By providing a perpetual stimulus to growth does it permit events to occur, such as mutations, gene rearrangements, or changes in the control of the expression of genes, which overcome the ability of the cell to police its own genetic stability?

Such is the nature of scientific problem-solving. Just as one set of questions has been posed, and some partial solutions encountered by experiment, new questions immediately reveal themselves. The study of lymphocyte immortalization should provide several generations the joy of this struggle. Such study has already had some useful by-products, among them a regular method to establish human cell banks for laboratory study of genetic disease and normal physiologic processes, and the possibility of making human monoclonal antibody from immortalized cells. No doubt further analysis of the intracellular pathogenesis of EBV infections will continue to offer up surprises and an occasional finding which is beneficial to human welfare.

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