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PREFACE

The importance of persistent viruses in human disease is well appreciated, but pathogenetic mechanisms have generally been poorly understood. With the recent advent of improved methods for study, many of these mechanisms have been definitively characterized. This meeting was organized to disseminate current information concerning viral persistence and its role in disease and to promote interactions between workers investigating diverse aspects of these agents. This meeting was the first since the 1974 ICN–UCLA Symposium on Mechanisms of Virus Disease to focus on pathobiological mechanisms in animal virology.

We wish to express our appreciation to the program participants, whose contributions to both the plenary and poster sessions made this meeting a success. We gratefully acknowledge the ongoing financial support from ICN Pharmaceuticals and a generous contract from the National Institutes of Health (National Institute of Neurological and Communicative Disorders and Stroke, National Institute of Allergy and Infectious Diseases, Division of Cancer Research Resources & Centers/National Cancer Institute, National Institute of General Medical Sciences, Fogarty International Center, Viral-Oncology Program/National Cancer Institute), which helped defray the invited speakers' travel expenses. Our special thanks go to Dr. Earl Chamberlayne of the Fogarty International Center for his efficient and helpful service in the capacity of Project Officer for the NIH contract. Finally, we are indebted to a talented collaboration of women, whose clerical and administrative expertise brought this meeting to fruition: Carolyn Hench, Kathy Morris, Bobbi Ottis, Brenda Seidman, Fran Stusser, and Donna Vratari.

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ALTERATION OF ESCHERICHIA COLI OUTER MEMBRANE PROTEINS BY PROPHAGES

A MODEL FOR BENEVOLENT VIRUS-CELL INTERACTION

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ABSTRACT Lambda lysogens of E. coli reproduce more rapidly than isogenic nonlysogenic strains when mixed populations are grown together in glucose-limited chemostats. Lysogens of Phages P1, P2 and Mu also show increased reproductive fitness when grown with the corresponding nonlysogens in glucose-limited chemostats. When the same strains are grown together in batch cultures, in which growth may be limited or not by the glucose concentration, lysogens and nonlysogens show the same reproductive fitness. The outer membrane proteins of bacteria grown in glucose-limited chemostats display a different composition from the same bacteria grown in batch cultures with glucose. In addition the presence of the prophage changes the proportion of the major outer membrane proteins when the lysogens are grown in chemostat cultures. It is suggested that the prophage can modify the membrane proteins of the bacteria by lysogenic conversion and enhance the fitness of the bacteria in certain environments. This seems to be comparable to the situation in animal cells, in which the growth and membrane properties are changed by viral infection or by induction of viral genes. A model is presented suggesting that oncogenes or proto-viruses may enhance the growth and development of animal cells under certain conditions. The widespread occurrence of viral genes in animal cells may have resulted from the natural selection of animal cells with increased reproductive fitness.

INTRODUCTION

Lysogeny is the process in which a temperate bacteriophage infects a bacterium without causing lysis and directs the integration of phage DNA into the chromosome of the bacterium. Thereafter, the phage DNA (the prophage) is repli-

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cated along with the bacterial DNA. The growth and physiology of the bacterial lysogen are indistinguishable from that of the non-lysogen except that the lysogen is immune to infection by homologous phage. This immunity has generally been used to explain the evolutionary selection of lysogenic bacteria (1).

Bacteriophages such as lambda, P2 and Mu all integrate their DNA into the E. coli chromosome. Lambda has one specific integration site and can use either phage or bacterial recombination enzymes to integrate the prophage DNA. P2 integrates at several chromosomal locations and is unusual in that the prophage is not inducible by ultraviolet light (2). Mu can integrate at many sites within any gene of the E. coli chromosome by "illegitimate recombination" that does not require the usual recombination enzymes. It has been pointed out that Mu lysogens have properties that are similar to eucaryotic cells infected by SV40 virus (3).

The stable association of phage and bacteria in lysogeny appears to be widespread in nature. Most bacteria isolated in nature possess several prophages as well as other extrachromosomal elements (4). In some cases it can be shown that the prophages modify the surface properties of lysogenic bacteria as well as confering immunity to infection by phage of the same type (5). The survival and reproduction of bacteria in nature depend on their ability to attach to specific surfaces or tissues. In other words, a bacterial colony must become established in a favorable ecological niche. It might be the mucosal lining of the intestine, a root on a legume, or a habitable rock in a river bed (6, 7, 8). The ability of bacteria to attach to surfaces by formation of a "glycocalyx" is often correlated with lysogeny (9). Moreover, there are specific examples of lysogenic conversion in which the prophage genome alters biochemical properties of the bacteria. The O-antigens in Salmonella anatum are changed by the phages ε^{15} and ε^{34}. Also harmless strains of Corynebacterium diphtheriae became toxin producers in the presence of phage β (5).

Most of the lysogenic bacteria survive in nature in harmony with their environments and prophage. In fact, the human body contains more bacteria in the intestinal tract than all the rest of the bodys' cells put together, and presumably we could not survive without these bacteria (10). To cause a disease a pathogen must multiply in host tissues, resist the host defenses and damage the host in some way (8). Bacteriophage may play a special role in the reproductive fitness of bacteria in particular environments by modifying bacterial colonization or enhance growth. According to this view the symbiotic association of prophages and bacteria is a consequence of natural selection and is clearly of evolutionary advantage to phage and bacterium. Not only do lysogenic

bacteria acquire immunity to infection by phage of a similar type but these lysogens may be reproductively more fit. Fitness, in this sense, refers to the contribution that lysogenic bacteria make to the gene pool of successive generations (11). The prevalence of lysogenic bacteria found in nature is in accord with these views.

We have shown that lambda lysogens of E. coli are more fit than non-lysogens when both strains are grown together in glucose-limited chemostats. In batch cultures at any glucose concentration both strains reproduce at identical rates (12, 13). Lysogens carrying the prophages P1, P2 or Mu are also reproductively more fit than non-lysogens in chemostats (14). The increased fitness of the lysogenic strains correlates with changes in the composition of the major outer membrane proteins.

These observations are similar to changes observed in the growth rates and membranes of animal cells transformed by viruses. A basis for these changes is found in the proto-virus and oncogene hypotheses (15, 16, 17). The association of viral genes with animal cells has a long evolutionary history (18, 19) and there is evidence that viral gene expression alters membranes (20, 21).

A model is presented suggesting that animal cells carrying viral genes or genomes result from the natural selection of cells with increased reproductive fitness in certain environments. In this view viral genes are as essential to cell survival as are cellular genes. Transformation and unregulated growth of cancer cells is an anomaly. It is perhaps analogous to the detrimental hyperimmune response of an animal to a harmless viral or bacterial infection (22).

RESULTS

Physiological Experiments. In order to test for the reproductive fitness of one organism versus another, the frequency of each organism in a mixed population must be measured. In the case of bacteria growing together in mixed populations, each genotype must be distinguishable from the others. We have devised techniques for comparing the relative fitness of lysogenic and non-lysogenic bacteria growing together under a number of different growth conditions.

Prophage are maintained in the integrated state in lysogenic bacteria by a repressor protein that binds to an operator site on the DNA and prevents expression of all prophage genes that would be required to synthesize phage particles. The maintenance of the prophage in the bacterial DNA requires the continuous presence of functional repressor proteins. Phage mutants can be isolated which produce a repressor pro-

tein that is thermolabile. Bacteria which are lysogenic for this class of mutant prophages are stable lysogens at low temperatures (25°C-30°C) but are induced to produce phage and are lysed at high temperatures (38°C-42°C). Mutants producing thermolabile repressor proteins have been isolated for the bacteriophages lambda, P1, P2 and Mu (13, 14).

It is a simple matter to determine if bacteria are lysogenic or not using these mutant phages. A lysogenic bacterium will produce a visible colony when plated on nutrient agar and incubated at 30°C, but will fail to produce a visible colony at 42°C since the bacterium will lyse. In a mixed population of lysogenic and non-lysogenic bacteria, the frequency of each type is easily determined. Bacteria are plated on nutrient agar plates and incubated at 30°C and 42°C. The number of colonies appearing at 30°C are a measure of the total number of bacteria in the population whereas the colonies appearing at 42°C measures the number of non-lysogenic bacteria. The number of lysogenic bacteria is the difference between these two numbers.

This method has been used to examine the relative reproductive fitness of isogenic lambda lysogens and non-lysogens. In these experiments an important control is to ensure that the phage are not transmitted from the lysogenic to the non-lysogenic bacteria. This is accomplished in the lambda lysogens by using lambda mutants that cannot be induced from the prophage state (ind$^-$). In addition, these lambda phage carry nonsense mutations in another gene essential for phage development (sus J), so that, in the rare instance that a prophage is excised, it cannot develop and produce viable phage, which can subsequently infect the non-lysogenic bacteria.

Figure 1 shows the results of an experiment in which a mixture of E. coli lambda lysogens and non-lysogens are grown together in either batch or chemostat cultures with limiting glucose as the energy source. In batch cultures both the lambda lysogen and non-lysogen reproduce at identical rates. Except for daily fluctuations the frequency of each strain in the population remains constant for at least forty generations. The same result is obtained in batch cultures with either limiting or excess glucose or with other carbon sources such as lactose or glycerol.

A very different result is observed when the lambda lysogen and non-lysogen are grown together in a glucose-limited chemostat. The lambda lysogen is reproductively more fit and rapidly becomes the most frequent strain of the population, reaching a frequency of 99.9% within 30-40 generations. It is interesting that in all the chemostat experiments that have been done, the non-lysogenic bacteria are never completely eliminated from the population although their frequency may become as low as 0.01%. The increased reproductive fitness of

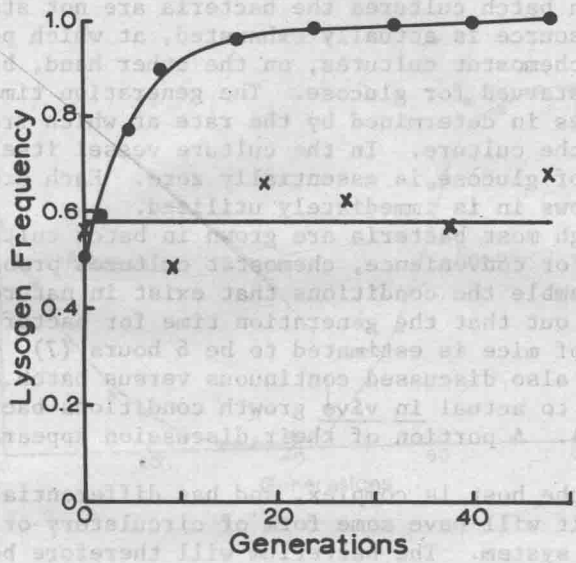

FIGURE 1. Reproductive fitness of the λ lysogen in batch and chemostat cultures. E. coli strain (AB) and the lysogen (ABλ) were grown together in either batch or chemostat cultures. Bacteria were grown in M9 minimal medium plus 0.01% glucose and 20 μg/ml methionine. The batch cultures were diluted 10^6 daily into fresh medium. The chemostat cultures were grown continuously with a generation time of 4 hours. Samples were plated daily on nutrient agar and incubated at 30°C and 42°C. Only non-lysogens give colonies at 42°C. x - - - x, frequency of ABλ in a mixed population of AB and ABλ growing in a batch culture; ● - - - ●, frequency of ABλ in a mixed population of AB and ABλ growing in a chemostat culture.

lambda lysogens in chemostat cultures also is observed for other carbon sources. The fitness does not depend on the generation time of the bacteria in the chemostat between generation times of 3-8 hours.

To fully appreciate these results it is important to understand the essential difference between growth in glucose-limited batch cultures and glucose limited chemostat cultures. In a glucose-limited batch culture (0.01% glucose) bacteria grow exponentially at the maximum generation time for the

particular medium until the glucose is exhausted. Thus, for glucose concentrations between 0.01% and 0.4% bacteria grow with the same generation time - only the final yield of cells varies. In batch cultures the bacteria are not starved until the carbon source is actually exhausted, at which point growth stops. In chemostat cultures, on the other hand, bacteria are constantly starved for glucose. The generation time in chemostat cultures in determined by the rate at which fresh medium flows into the culture. In the culture vessel itself the concentration of glucose is essentially zero. Each drop of medium that flows in is immediately utilized.

Although most bacteria are grown in batch cultures in the laboratory for convenience, chemostat cultures probably more closely resemble the conditions that exist in nature. Smith has pointed out that the generation time for bacteria growing in the gut of mice is estimated to be 6 hours (7). Woods and Foster have also discussed continuous versus batch cultures as they relate to actual in vivo growth conditions bacteria encounter (23). A portion of their discussion appears below.

> "When the host is complex, and has differentiated tissues, it will have some form of circulatory or translocatory system. The bacterium will therefore be in an environment which is constantly renewed in the sense that there will be, due to the activities of the host, both a continual replenishment of growth metabolites and a continual removal of waste products of metabolism. The situation is essentially similar for organisms living in an intestinal tract. The system is therefore analogous in many ways to continuous culture and less like the batch cultures that are the source of so much of the bacterial material used for metabolic studies in vitro."

Having shown that lambda lysogens of E. coli are reproductively more fit in continuous chemostat cultures, we tested the generality of the phenomenon. E. coli strains lysogenic for the phages P1, P2 and Mu were grown together with the isogenic non-lysogenic strains. In each case it was found that in batch cultures the reproductive rates were identical whereas in chemostat cultures the lysogens reproduced more rapidly (14).

Further conformation of the fitness of lysogenic bacteria in chemostat cultures was obtained by finding a condition in which the relative fitness could be reversed. Usually all cultures are grown aerobically. However, when the chemostat cultures are maintained under anaerobic conditions, the non-lysogenic bacteria reproduce more rapidly than the lambda lysogens. Figure 2 shows the result of such an experiment. A mixed population of the lambda lysogen and non-lysogen is

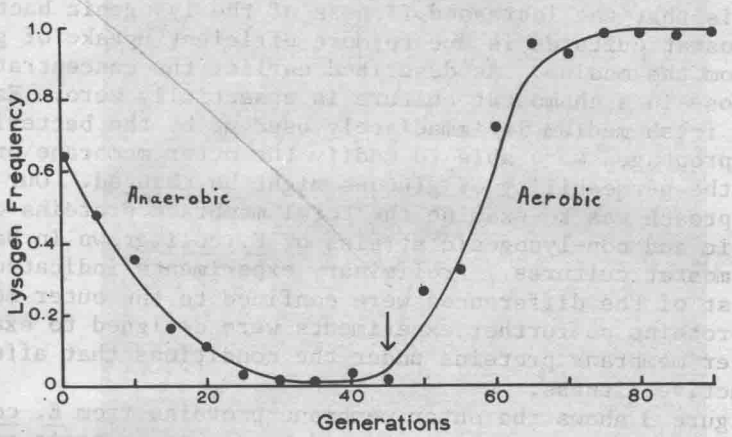

FIGURE 2. Reproductive fitness under aerobic and anerobic growth. E. coli strain (AB) and the lysogen (ABλ) were grown together in a glucose limited chemostat (0.01%) under anaerobic conditions. A mixture of N_2-CO_2 (95%-5%) was bubbled through the culture. After several days of anaerobic growth, the culture was switched to aerobic growth as indicated by the arrow. The frequency of the lysogen and non-lysogen in the population was measured by plating samples on nutrient agar and incubating the plates at 30°C and 42°C. Only the non-lysogens give colonies at 42°C.

grown anaerobically in a chemostat culture. When the frequency of the lambda lysogen has fallen to about 1%, the chemostat is switched to aerobic growth. The lysogenic bacteria reproduce more rapidly and quickly predominate in the population.

Biochemical Experiments. What is the biochemical basis for the increased reproductive fitness of lysogenic bacteria which are growing aerobically in glucose-limited chemostat cultures? Since the same concentration of glucose is presumably equally available to lysogenic and non-lysogenic bacteria, a difference in growth rate must reflect increased uptake of glucose or more efficient utilization.

The outer membrane of E. coli and S. typhimurium has been shown to regulate the permeability of the bacterium to molecules of particular sizes. The proteins that are involved in this process are the most abundant proteins in the outer membrane and have been termed "porins" (24, 25, 26). Our hypothesis is that the increased fitness of the lysogenic bacteria in chemostat cultures is due to more efficient uptake of glucose from the medium. As described earlier the concentration of glucose in a chemostat culture is essentially zero. Each drop of fresh medium is immediately used up by the bacteria. If the prophages were able to modify the outer membrane proteins, the permeability of glucose might be changed. Our initial approach was to examine the total membrane proteins of lysogenic and non-lysogenic strains of E. coli grown in batch and chemostat cultures. Preliminary experiments indicated that most of the differences were confined to the outer membrane proteins so further experiments were designed to examine the outer membrane proteins under the conditions that affect reproductive fitness.

Figure 3 shows the outer membrane proteins from E. coli strain ABλ which have been separated by electrophoresis on a SDS-polyacrylamide gel. The outer membrane proteins displayed in column A are from lambda lysogens growing exponentially in batch culture with excess glucose. The same pattern of protein bands is obtained when the bacteria are grown in batch culture with limiting glucose (unpublished results). The non-lysogen also gives the same pattern when grown in batch cultures. That is, both lysogenic and non-lysogenic bacteria have identical outer membrane protein compositions if grown in batch cultures.

Column B shows the outer membrane protein from the lambda lysogen grown in a glucose limited chemostat. The relative amounts of the various outer membrane proteins is quite different when the lysogen is grown in batch or chemostat cultures. Precisely the same differences are observed if the non-lysogen is grown in batch or chemostat cultures with one significant exception. The most abundant outer membrane proteins are labeled I and II in figure 3. Actually band I consists of 2 proteins which are poorly separated. When the lambda lysogen is grown in a glucose-limited chemostat, protein II is reduced in amount by 40%-50% as compared to the amount found when the non-lysogen is grown in the chemostat (column B, figure 4; and unpublished results). In general, when the non-lysogen is grown in the chemostat or in batch culture, the amounts of protein I and II are identical. If the lambda lysogen is grown in the chemostat, the amount of protein II is always markedly reduced (column B, figure 3). This change in the relative abundance of the major outer membrane proteins correlates with the increased fitness that is observed for the