Dr. Spiegelman responded to questions on his paper by Dr. Melnick. His group tested only tumor tissues from Burkitt’s lymphomas and not lymphoblastoid cell lines derived from such tumors. Only limited attempts have been made to evaluate tissues from other diseases that are associated with lymphoreticular proliferation. Results with acute phase, mononuclear cells from one case of infectious mononucleosis were negative, but this finding obviously needs confirmation by study of additional cases. Carcinomas of various origins (e.g., lung, stomach, colon, rectum, ovaries) and brain tumors were positive for oncornavirus information. Studies with cervical carcinomas, which were studied only recently, and a case of sarcoidosis are still incomplete, and lepromatous leprosy has not been studied.

To questions by Dr. Goodheart, Dr. Spiegelman replied that he has not yet examined animal tumors caused by herpesviruses but that such studies are planned.

Dr. Deinhardt asked whether any checkerboard hybridizations between murine leukemia, murine mammary tumor, feline, and other mammalian C-type viruses had been performed and if much overlap was found. This would be important because apparently in some experiments much less cross-hybridization was found between nucleic acids of human tissues and feline C-type viruses than had been reported for murine viruses and human tissues; this finding needs clarification. Dr. Spiegelman stated that these specific experiments had not been done but such questions would be resolved as work in this area progresses. Probes that are used almost routinely today are made with “particles” found in human tissues, and hybridization between these probes and nucleic acids of human neoplastic tissues occurs at a much higher rate than ever observed with probes made with murine C-type viruses. It has been shown that actinomycin D, which was always added to the reaction mixtures to prevent DNA-directed DNA synthesis, resulted in the copying of much more of the genome. We had always assumed, Dr. Spiegelman said, that we were copying only 10% of the viral gene, but we now know that the probes used were actually much better than that. Yet there are some strange phenomena. RD-114 virus, which is probably of cat and not human origin, hybridizes extremely well to cat DNA but virtually not at all to human DNA. However, probes made with RD-114 hybridize better with nucleic acids from Hodgkin’s disease tissues than with probes made from Rauscher murine leukemia virus. We also found distinct differences between murine leukemia and sarcoma viruses. The meaning of many of these results is currently unclear, and very careful quantitative and qualitative hybridization experiments are needed for a full understanding of these findings.

Dr. Benyesh-Melnick asked whether DNA made with 70 S RNA and reverse transcriptase found in nasopharyngeal carcinoma would back-hybridize to Epstein-Barr virus DNA, as she felt this would be at least an interesting control. Dr. Spiegelman stated that such experiments are planned in collaboration with Dr. zur Hausen.

Dr. Nahmias asked Dr. Spiegelman to speculate on how the findings on DNA and RNA viruses in Burkitt’s lymphoma, cervical carcinoma, and other neoplastic diseases would ultimately all fit together. Dr. Spiegelman declined and commented that more data are needed, that the experimental road to a further understanding is clear, and that, at this time, not enough is known to speculate very fruitfully. He also made a comment on the search for C-type information. In general, electron microscopy will detect particles only if they are present in profusion. The use of serological approaches assumes that the relevant antiserum is available. For example, one would certainly miss RD-114 if one depended only on using the antiserum against the gs antigen of feline leukemia virus. The most sensitive method available at present is the simultaneous detection test. The latter has the further advantage that it is independent of prior experience with the particular virus being detected. Once a positive result is obtained, the DNA synthesized can be used as further tool via molecular hybridization.

There was a final question from the floor concerning the data Dr. Spiegelman presented earlier, which indicated that the “virogene” theory may not be generally applicable. Dr. Spiegelman was asked if he wished to imply that leukemia could be an infectious disease, despite all other evidence which indicates that leukemia is in classical terms not an infection from the outside and that “normal cells” can be induced to produce typical C-type viruses by various physical or chemical treatments without infection from the outside.

Dr. Spiegelman concluded the discussion of his work by pointing out something that he felt was relevant. It is very important to understand that the provirus and the virogene hypotheses are not mutually exclusive. He feels that they could both be true, and that it might well depend on the biology of the system under study. His “personal bias” is as follows and is supported by some factual data. Some inbred animals seem to satisfy in fact the virogene hypothesis, and it may well be that the evidence supporting this is possibly a laboratory artifact, as he has been working with a special group of animals that were inbred for particular purposes.
We know that viral genetic information can get into the cell genome and it then can pass in the germ line of the animals. This is the virogene hypothesis. His evidence suggests, however, that this hypothesis may not be true for randomly bred populations, and as far as infectivity from the outside is concerned, he does not think that this is automatically implied when he states that the viral genetic information is not present in the genome of every cell; it may exist as an extra-chromosomal component in a few cells of the animals.

Dr. Munk replied to a question on his paper by Dr. Klein that he had not yet determined whether his transformed carrier cultures could be cured by the addition of antibodies. Dr. Gerber asked Dr. Munk whether he had tried to transform primary human lung cells instead of selected cell populations that could be considered as already transformed at the start of the experiment. Dr. Munk stated that no experiments had been performed with primary human lung cells but that the experiment had been repeated several times with identical results with the cell populations described in his paper, which he did not consider to be transformed. Only infected cells established permanent cell lines, whereas the control cultures always stopped growing after 35 to 45 cell culture passages. On the other hand, similar experiments with rodent cells always failed and no transformed cell lines were established. Dr. Munk speculated that the alteration of the host-cell-virus relationship by elevated incubation temperatures may be specific for human cells.

Dr. Falk asked whether the transformed cell lines of Dr. Rapp were producing interferon and whether the tumors in the transplantation experiments consisted of donor or of recipient cells. Dr. Rapp replied that no detectable amounts of interferon were produced by the transformed cells and that the tumors consisted of donor cells in the experiments with the first transformed cell line in which chromosomal analyses were performed.

Dr. Henle reported his failure to stain viral antigens by the indirect fluorescent antibody test in one of Dr. Rapp's transformed cell lines with human sera obtained from patients with cervical carcinoma, herpetic cervicitis, and other diseases, despite the fact that all sera stained herpesvirus antigens in herpesvirus-producing control cultures.

Dr. Rapp confirmed that he had obtained irregular results with human anti-herpesvirus sera and that he had been most successful in staining viral antigens in the transformed cells by using hamster sera made in the same inbred line of hamsters used for the transformation and tumor transplantation experiments. In addition, the sera of tumor-bearing hamsters reacted well with altered cell surface antigens and contained neutralizing antibodies (titer of 1/5 to 1:40 in a plaque reduction test against 100 to 1000 plaque-forming units of virus) to herpes simplex virus type 2 and no or only very low titered levels of antibodies to type 1.

Dr. Munk added that he used rabbit sera and that sera of the same rabbits obtained before immunization were always included as controls.

Dr. Nahmias also had used Dr. Rapp's transformed cells and had obtained membrane fluorescence in about 50% of the cells with rabbit anti-herpes simplex type 2 sera, but only <5% of the same cells could be stained with anti-type 1 sera. He also tried to stain the transformed cells with human sera from patients with cervical cancer or overt herpes simplex virus type 1 or type 2 infections. Indirect tests with labeled anti-IgM, IgG, or IgA were performed. Irregular patterns were obtained, but Dr. Nahmias pointed out that their results were not as negative as Dr. Henle's: 30% of their sera were positive and they obtained the highest percentage of positives in those tests in which anti-IgM was used.

Nevertheless it was clear that the difficulty in detecting viral antigens with human sera, which identifies such antigens easily and consistently in productively infected control cells, is puzzling and needs further study.

Dr. Roizman added some data concerning some experiments that were done by Dr. Kieff at the University of Chicago; he stressed the following points.

1. In one series of experiments in which a line of HSV-2-transformed hamster fibroblasts, obtained from Dr. Rapp, were cocultivated with HEP-2 cells, Dr. Kieff has observed eventual plaque formation on HEP-2 cell monolayers. The "plaque"-forming ability of the cocultivated cells on HEP-2 cells was not decreased by freezing and thawing. By thin sectioning in our laboratory, the cells in these plaques look like cells that are being destroyed by herpesviruses with typical margination of chromosomes. Very few virus particles can be seen. Fluorescent, labeled rabbit antisera against HSV-2 stain plaques quite heavily. The production of virus on HEP-2 cells is quite low and slow. Therefore, we believe that the virus may be defective. He reiterated that this is as yet only one series of experiments.

2. With regard to detection of HSV-2-specific RNA in the transformed hamster cells, the following can be said. First, there is some hybridization of labeled hamster transformed cell RNA to HSV-2 DNA but it is slight. Second, we are in the process of trying to determine just how much of the genome is transcribed in these transformed cells. These experiments require large amounts of transformed cell RNA for precision. In preliminary experiments the amount of virus-specific RNA was so low that we do not have an end point.

Dr. Deinhardt asked Dr. Rapp to comment on this, and Dr. Rapp replied that he did not wish to answer since he and Dr. Kieff are doing the work together. Cocultivation and fusion experiments done in the laboratory using rabbit kidney and human embryonic kidney cells both of which are susceptible to HSV-2 infection have failed to yield virus.

Dr. Benyesh-Melnick said that Dr. Rapp's immunization experiments using the hamster cells transformed by herpes simplex virus were very interesting and intriguing and asked whether antigens other than SV40 and herpesvirus had been used and whether similar experiments had been carried out with cells transformed by other viruses. Dr. Rapp replied that both adenovirus type 12 and irradiated SV40 have been used without success. Neither prevented metastases by cells transformed by herpesvirus. Many of the cells transformed by other viruses did not metastasize. However, primary
transplants of cells transformed by SV40 or by the SV40-adenovirus hybrids were not affected by prior immunization of the animals with herpesviruses.

Dr. Auersperg and Dr. Nahmias then discussed their experience with cervical cancer cell lines and herpes simplex viruses. Dr. Auersperg commented that in her laboratory they have been trying to establish cell lines of human invasive cervical carcinomas in culture, which would not de-differentiate to the point that has been reached: e.g., by HeLa cells, but rather cell lines that would retain some of their initial properties and therefore might be more representative of the original tumor. A few such lines were established, and the properties of one of these, line C-4 11, are briefly summarized.

The C-4 11 cell line, she continued, originated from the biopsy of a very poorly differentiated, small-cell, squamous carcinoma. Cytogenetically, one of the distinguishing features of the cell line is the fact that it has a hypodiploid karyotype which has remained constant for several years in culture, during which time the cell number doubled every 24 to 48 hr. The chromosome stem line is 44 to 45, and although there are a number of numerical anomalies such as deficiencies in groups A and E, the majority of chromosomes can still be classified within the normal human karyotype. In addition to this relative stability of the karyotype, the cells have retained functional and morphological properties that are normally associated with basal cells in stratified squamous epithelium. When they are grown as tumors in hamster cheek pouches, they infiltrate host tissues characteristically in small groups that are surrounded by basement membranes, as identified electron microscopically between the fibroblasts and the cancer cells. Basement membranes are produced in this case by malignant cells, after a number of years in culture, although these structures are commonly considered to be associated with the differentiation of normal basal cells and to disappear in carcinogenesis or in prolonged culture.

Dr. Auersperg went on to say that, in vitro, the C-4 11 cells form epithelial colonies and they show a striking tendency to remain monolayered, even when crowded. However, they are not contact inhibited. Like most tumor cell lines, they continue to proliferate when crowded, but they have their own way of getting around the resulting problems. As seen in vertical sections through cultures of increasing cell density, the cells are initially oriented horizontally in a confluent monolayer, with no overlapping whatsoever. If they are allowed to continue to proliferate, they change from a horizontal to a vertical orientation, become cuboidal or columnar and, if they still continue to multiply, they begin to separate from one another and shed into the culture medium. They have another rather interesting way of getting around crowding while remaining monolayered. Confluent cell groups lift off the glass surface and buckle, so that these groups appear as hemicysts in an essentially monolayered culture.

Electron microscopically, several distinct cell surface regions can be identified on vertical sections through C-4 11 cultures. In the regions adjacent to culture medium, adjoining cells are connected by junctional complexes, which are highly specialized surface structures found in normal basal cells, for example, in fetal epithelia. In addition, if C-4 11 cells are stained for acidic cell surface mucoproteins, more specializations can be seen. Thus the distribution of mucoproteins on the surface exposed to the culture medium is quite different from that of the surface exposed to intercellular spaces, particularly in association with filamentous structures within the cell coat. The structural differentiation is probably associated with functional differentiation between various cell surface regions and might also be of importance in influencing the special distribution of cell surface antigens.

Dr. Nahmias then added that they have been interested in working with Dr. Auersperg's cervical cancer cell lines because they believed them to be more representative than HeLa cells. Briefly, their results with the C-4 11 cell lines are as follows.

1. HSV type 1 or 2 strains grew slowly in a focal fashion, so that cell monolayers were only destroyed by the virus in two weeks; virus titers were about two logs less in these cells than in HEp-2 or rabbit kidney cells.

2. About 10 to 15% of the cervical cancer cells demonstrated membrane fluorescence with fluorescein-conjugated anti-HSV-2 sera, and about the same number of cells showed positive membrane potential when transmembrane potential records were made; in comparison, HEp-2, rabbit kidney, L-cells, human fibroblast and a number of other primary cell cultures or cell lines of various species showed negative potential.

3. They have been unable to demonstrate infectious herpes simplex virus in these cells by a variety of methods, including cocultivation, X-irradiation, or use of iododeoxyuridine or alkaline pH.

Dr. Aurelian replied to questions by Dr. Deinhardt, Dr. Falk, and Dr. Melnick as follows.

1. So far her group has tested only 6 tumor biopsies for presence of AG-4. Four of these were squamous carcinomas and 2 were adenocarcinomas of the cervix. The antigen was present in 3 of the 4 squamous carcinomas and in none of the adenocarcinomas.

2. The titers of antibody to AG-4 are generally low. In the patients whom her group has studied, they did not see any correlation between the preinvasive or invasive nature of the lesion and the titer of antibody to AG-4.

3. All sera were tested against both AG-4 and AG-H antigens. Furthermore, all sera positive for both antigens were considered negative for AG-4 and were not incorporated in the study. A very small number of sera fell into this category. A serum positive for AG-H only was recorded accordingly. The zeros in Tables 4 and 5 mean that there was no reactivity with AG-H.

4. The complement fixation test we used in this study is a modification of the original Wasserman and Levine test (2). It has been used extensively [as stated by Dr. Gerber and by Marcus and Townes (1)]; it is very sensitive and is of particular value in the identification of low-avidity antibody. We also tried two other complement-fixation tests; the big volume (7.5 ml) and the microtiter complement fixation tests both with negative or nonreproducible results.
Dr. Aurelian's neutralization test does not determine the titer of neutralizing antibody in patient sera but rather the presence or absence thereof. On the other hand, Dr. Thiry's study describes a decrease in the titer of neutralizing antibody to HSV-2 following hysterectomy and not the complete disappearance of the antibody. Therefore, there does not seem to be any contradiction between the data of the two investigators.

Dr. Gerber reemphasized from his own experience the sensitivity of the complement-fixation test used by Dr. Aurelian. He stated that this test is exceedingly sensitive and that one must guard against false-positive results. For this reason it has been abandoned for routine tests but, as a research tool under appropriately controlled conditions, it can give excellent results with high sensitivity.

References

Summary of Informal Discussion of Experimental Approaches

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