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POLYKARYOCYTOSIS INDUCED BY VIRUSES

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Measles, varicella, the herpesviruses, some myxoviruses, and several other agents induce polykaryocytosis *in vitro* and some also *in vivo*. The polykaryocytes are formed by fusion of mononucleated cells and may contain thousands of nuclei. The purpose of this communication is to present evidence that (1) the cells fusing into polykaryocytes must be functional but different in some phenotypic characteristic and (2) the phenotypic difference is a result of an alteration in cell structure caused in some manner by viruses.

The Genesis of Polykaryocytes.—The evidence that polykaryocytes arise by fusion of cells differing in some phenotypic characteristic emerges from studies of the effect of *herpes simplex* virus, strain MP, on FL and HEp-2 cells.[1-3] In cell cultures exposed to less than 0.01 plaque-forming unit of virus per cell, polykarocytes are the predominant feature. On the other hand, in cell cultures exposed to sufficient virus to infect all or most cells, polykaryocytes are scarce and contain few nuclei. Rounded mononucleated cells showing typical inclusions and containing viral antigen predominate. These findings cannot be explained on the basis of heterogeneity of viral population or cytotoxic effects of concentrates, since the progeny of

virus cloned repeatedly behaves in exactly the same way and, moreover, the yield of virus is actually higher if all cells are infected initially. It must be concluded therefore that polykaryocytes arise by fusion of infected and uninfected cells, or cells differing in the time of infection. In accord with this hypothesis, cultures exposed to small amounts of virus and examined after several cycles of virus replication have elapsed show both polykaryocytes and degenerated mononucleated cells. On the other hand, if the extracellular virus progeny of cells infected initially is prevented from reaching uninfected cells by the addition of antiviral antibody to the culture fluid, only polykaryocytes develop.[1] The effectiveness of antibody in preventing the spread of virus through the extracellular fluid is evident from the fact that the count of polykaryocytes is proportional to virus concentration or equal to the number of infected cells seeded on the monolayer of uninfected cells.[4]

Since each cell has its own membrane, a mere dissolution of the membranes would lead to spillage of cytoplasmic contents into the extracellular fluid. It may be postulated that polykaryocytes are formed in two steps. First, the altered membrane of the infected cell fuses with the normal membranes of uninfected cells forming a *propolykaryocyte*. Second, the fused membrane is probably less cohesive, is ineffective as a barrier, and eventually undergoes dissolution giving rise to a true polykaryocyte.

The existence of a fused membrane is apparent from the finding[1,5] that young (24 hr old) polykaryocytes are broken up by trypsin into elements numerically equivalent, roughly, to the number of their nuclei, each capable of inducing a new polykaryocyte. Since debris of infected cells is ineffective in inducing polykaryocytes,[6] these elements must be whole cells. Older (48–72 hr) and much larger giant cells treated with trypsin yield very few polykaryocyte-inducing elements, indicating that the fused membranes partitioning the propolykaryocyte have disappeared. It is probably not a coincidence that in young polykaryocytes the nuclei are in relatively the same position as they were prior to fusion, whereas in older polykaryocytes they migrate and clump.[1–3] Colchicine at concentrations sufficient to cause mitotic arrest appears to cause a delay in the dissolution of fused membranes. In cultures treated with colchicine 24 hr after infection, the polykaryocytes increase in size until virtually all the interphase cells are recruited, but there is no migration or clumping of nuclei. In the presence of the drug, arrested mitotic cells are not recruited into polykaryocytes.[3]

Some of the events taking place in the course of the formation of polykaryocytes may be deduced from three observations. First, propolykaryocytes fuse on contact. This observation suggests that the outer membrane of the propolykaryocyte must be normal at some stage if it is to fuse with an altered membrane of another propolykaryocyte. Second, it has been observed[4] that the highest rate of recruitment of cells into polykaryocytes is at or near 37°C, which is the optimal temperature for release of virus from infected cells into the extracellular fluid.[7] This temperature, however, is supraoptimal with respect to total viral yield; several times larger yields may be obtained from infected cell cultures incubated at 34°C (see ref. 7). It may be deduced from these studies that the rate of recruitment of new cells by the propolykaryocyte depends on the rate of egress of virus from infected compartments into the outer uninfected compartments and not on the absolute concentration of virus in the propolykaryocyte. Finally, it has been observed that in large

polykaryocytes the nuclei in the center migrate and clump whereas those at the margins are scattered.[1, 2]

On the basis of these observations, the recruitment of cells into polykaryocytes may be visualized as follows: On fusion of an infected cell with uninfected cells, the newly formed propolykaryocyte consists of several uninfected compartments arranged radially around an infected compartment. The outer membrane of the uninfected compartment is unaltered. As the virus streams from the inner compartment through the fused membranes and the replication of virus begins in the outer compartments as well, the outer membrane of the propolykaryocyte becomes altered and more uninfected cells are recruited. The process continues as long as there are uninfected cells bordering the polykaryocyte. In large cells containing several thousand nuclei, the center may be a true polykaryocyte, whereas the margin is still in the propolykaryocyte stage.

There are indications that the polykaryocytes induced by measles virus form in a similar fashion. Infection of cell cultures with measles virus (Edmonston strain) is manifest in the formation of long, irregular cytoplasmic processes, rounding of mononucleated cells, and polykaryocytosis.[8] In young (2–4 day old) polykaryocytes, the nuclei are scattered in an irregular fashion throughout the cytoplasm; in older polykaryocytes, the nuclei migrate and clump.[9] Reissig *et al.*,[10] confirmed by Frankel and West,[11] noted that the addition of glutamine to a deficient medium caused infected cell cultures to develop fewer polykaryocytes; mononucleated degenerated cells predominated. Seligman and Rapp[12] found that after several passages of the Edmonston strain, mononucleated cells predominated. They also isolated a strain of virus which caused only polykaryocytes "as its specific effect at end-point dilution" and which was not affected by glutamine. A clue to the puzzling behavior of the Edmonston strain in the hands of different workers emerged from the report by Oddo *et al.*[13] These workers found that HeLa cell cultures infected with measles virus passed serially at 1:1 dilution showed predominantly mononucleated degenerated cells. However, after 64 passages in undiluted form, the virus rapidly recovered its ability to induce polykaryocytosis when repassaged at 1:100 dilution. In the light of the experience with *herpes simplex* virus, it is clear that if the dose of measles virus is sufficient to infect all or most cells parasynchronously, the mononucleated cells predominate. If only a fraction of the cells is infected, polykaryocytes appear. The measles strain of Seligman and Rapp may indeed be a mutant of the Edmonston strain with reduced yield, rate of replication, or rate of release from infected cells. Finally, the role of glutamine could be that of an essential nutrient required for high virus yields. In the absence of glutamine, the yield may be smaller, fewer cells are infected after each virus cycle and consequently polykaryocytes predominate.

Cell Integrity and Function in Relation to the Genesis of Polykaryocytes.—The results of several studies indicate that in order to fuse, cells must be physically intact and functioning. Cells which take up trypan blue, i.e. "dead" cells, do not fuse.[14] In another study,[6] it was found that HEp-2 cells infected with MP virus fail to initiate polykaryocytosis after immune injury by rabbit anti HEp-2 serum and complement. Injured infected cells and debris of infected cells are not effective in inducing polykaryocytosis.

Recently, some information concerning the extent of participation of infected

and uninfected cells in the genesis of polykaryocytes emerged from two studies which are described and jointly discussed below.

In the first study, mouse embryo cells grown in monolayer cultures were suspended with trypsin 2 hr after exposure to MP virus, washed with antiviral antibody, diluted, and seeded on monolayer cultures of mouse embryo and HEp-2 cells, respectively. The counts of polykaryocytes formed on the monolayer cultures of HEp-2 cells and mouse embryo cells were identical. In the reciprocal experiment, infected HEp-2 cells induced the same number of polykaryocytes in monolayer cultures of HEp-2 cells and mouse embryo cells. However, in a simultaneous titration, free MP virus induced nearly twenty times more polykaryocytes in cultures of HEp-2 cells than in cultures of mouse embryo cells.

In the second study,[15] HEp-2 cells grown on monolayer cultures were exposed immediately and 24, 48, and 72 hr after X-irradiation (100–4,000r), respectively, to free MP virus or were seeded with cells suspended with trypsin 2 hr after infection with MP virus. In the experiments with free MP virus, it was found that in cultures infected immediately after irradiation the polykaryocyte count was independent of the dose of X-irradiation. If the exposure to virus was delayed for 24 hr, the polykaryocyte count in X-irradiated cultures was lower than in untreated cultures. The difference in counts was more pronounced the longer the infection of irradiated cells was delayed. Moreover, the decrease in the number of polykaryocytes was dependent on dose of X-irradiation. From the results of several experiments, it was estimated that between one and two hits were sufficient to render a cell incapable of initiating a polykaryocyte. The lower counts obtained with free virus in X-irradiated cell cultures could not be attributed to a difference in the rates of absorption of virus to infected and X-irradiated cells. In contrast to the results of experiments with free virus, it was found that the polykaryocyte counts obtained after seeding X-irradiated HEp-2 cell monolayer cultures with infected untreated HEp-2 cells were independent of X-irradiation dose or delay (0–48 hr) after irradiation.

The results of both studies indicate that infected cells are more efficient in inducing polykaryocytes than free virus. They also indicate that a distinction must be drawn between the infected cells which initiate polykaryocytosis and the uninfected cells with which they fuse. The infected and uninfected cells do not participate equally in the process of fusion, but rather the infected cell "recruits" the uninfected cells. This is evident from the observation that cells inherently less susceptible to infection with free virus, or rendered incapable of becoming recruiters by X-irradiation, may still be recruited into polykaryocytes. The recruitment of cells, however, is not indiscriminant. Mitotic cells, particularly at metaphase are resistant to recruitment.[3]

The Role of Viruses in the Genesis of Polykaryocytes.—Many diverse agents, notably mycobacteria, lipids, and lipophilic substances induce polykaryocytosis both *in vivo*[21] and *in vitro* (a comprehensive bibliography may be found in reference 22). Viruses therefore have no unique ability to induce polykaryocytosis. A common characteristic of polykaryocytosis-inducing viruses is that they contain lipids, since they are rapidly inactivated by ether. However, ability to induce polykaryocytosis is not a general property of ether-sensitive viruses. Moreover, the mP strain of *herpes simplex* virus, which does not induce polykaryocytosis,[1]

cannot be differentiated from the MP strain on the basis of ether or chloroform inactivation rates (to be published).

It has been postulated that polykaryocytes are formed by fusion of cells damaged by a cytolytic noninfectious viral subunit similar or identical to viral hemolysin.[16, 17] In this connection, it is of interest that concentrated parainfluenza,[14,18] measles,[19] and vaccinia[20] viruses sterilized by U.V. light induce polykaryocytes in Ehrlich's ascites cells and FL and L cell cultures, respectively, within 1 to 6 hr after exposure. The results of these experiments, however, are not adequate proof that the site of action of these preparations is in the cell membrane or that hemolysis and polykaryocytosis induced by viruses are similar processes. It should be noted that antiviral antibody mixed with the concentrates prevents polykaryocytosis. The antibody is ineffective if added after exposure of cells to the virus. The action of the virus, therefore is not at the cell surface accessible to antibody. Moreover, herpesviruses do not appear to lyse erythrocytes.

There is some evidence which might be interpreted as suggesting that the site of primary damage leading to polykaryocytosis is not necessarily at the cell membrane but could be elsewhere. First, it has been reported[23] that Chinese hamster cells are resistant to infection with a strain of *herpes simplex* virus as evidenced by low virus yields and seemingly unimpaired ability of these cells to divide after being exposed to the virus. However, chromosomal abberrations were found in greater numbers in infected cultures than in controls. The authors deduced from these studies that the chromosomal abberrations were caused in some manner by the virus. Second, it was observed[3] that in interphase HEp-2 cells infected with the MP strain of *herpes simplex*, viral antigen appears first at the nuclear membrane and subsequently accumulates in the cytoplasm. In mitotic cells, virus appears and accumulates in the vicinity of chromosomes even after a nuclear membrane is formed. These observations suggest that in the nuclei of interphase cells as well, MP virus matures near the marginated chromosomes and migrates into the cytoplasm. Finally, the failure of X-irradiated cells to become recruiters may be due to one of two possibilities. Either the virus does not cause the specific alteration in cell structure because the structure has been preemptied by X-irradiation, or the primary damage at some distant site does not become manifest in the cell membrane. Some information concerning the structure damaged by X-irradiation may be deduced from the available data. The post-irradiation delay in the manifestation of the impaired capacity of these cells to becoming recruiters and the X-irradiation dose-response relationship are all compatible with the hypothesis that the structure damaged by X-irradiation is part of the cell genome. Taken together, these three observations may be interpreted as indicating that the multiplication of the MP virus interferes with some function of the cell genome and may damage it. This hypothesis, while interesting, is not supported by adequate evidence and may have to be abandoned.

In essence, adequate data is lacking concerning the primary site damaged by the polykaryocyte-inducing viruses and causing cells to become recruiters. It has been noted that mP and MP variants of *herpes simplex* virus differ in ability to induce polykaryocytosis; the events leading to fusion of cells into polykaryocytes may well emerge from studies of these mutants.

Studies of polykaryocytosis induced by viruses may elucidate the general prob-

lem of polykaryocytosis. It may be profitable to consider the possibility that all polykaryocytes arising by fusion of cells are formed in a similar fashion even though the primary lesion caused by various agents may be different. It is of interest for example that propolykaryocytes and true polykaryocytes resemble in appearance the "foreign body giant cells"[21] and the classical Langhans cells,[24] respectively. Finally, it has been reported recently[25, 26] that mixtures of somatic cells of different karyotype produce cells of mixed karyotype. It may well be that surface differences between partners is a prerequisite for the hybridization of mammalian somatic cells.

Summary.—The conditions required for the induction of polykaryocytes by *herpes simplex* virus and measles virus and some properties of these multinucleated cells suggest that they arise by fusion of functioning cells differing in some phenotypic characteristic. It is postulated that polykaryocyte-inducing viruses cause some disturbance in cells manifest in an altered cell membrane. The altered membrane of the infected cell fuses with membranes of uninfected cells giving rise to a propolykaryocyte. True polykaryocytes arise after the fused membranes dissolve and are characterized by clumped nuclei. Infected and uninfected cells do not participate equally in the process of fusion as evident from the fact that uninfected cells which lost capacity to initiate polykaryocytes may still fuse with a polykaryocyte initiated by another cell. The mechanism by which viruses induce polykaryocytes is not understood. Attention has been drawn to the fact that a variety of other, nonviral agents induce "foreign body giant cells" or Langhans-type giant cells which resemble the propolykaryocytes and true polykaryocytes, respectively, characterized in this communication.

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RELATIVE ANTIBODY-FORMING CAPACITY OF SPLEEN CELLS AS A FUNCTION OF AGE

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Our past studies have shown that the *in vivo* culture model is quite suitable for assessing quantitatively the antibody-forming capacity of lymphoidal cells[1–4] That is, a linear $\log_2$ relation with a slope of 1.0 can be demonstrated between activity, as expressed in terms of a 6-day agglutinin titer, and cell number. Using this model, we found that there exists in the intact mouse an autoregulatory mechanism that permits only a fraction of the total population of competent cells to participate in a given immune response.[4] Thus, for example, the cells from one-tenth of a spleen from a preimmunized mouse transferred into the irradiated mouse can be shown to produce as much antibody in response to an optimum secondary dose of antigen as can an intact mouse. In view of this autoregulatory mechanism, it would seem that data obtained from studies on the antibody-forming capacity of individuals throughout their life span (e.g., see the comprehensive study of Wolfe and his coworkers[5]), although informative, could not be readily interpreted. Preliminary studies were therefore carried out using the *in vivo* culture model to determine the primary antibody-forming capacity of 76×10^6 spleen cells (anti-sheep RBC response) from (C3H/Anf Cum ♀ × 101/Cum ♂) donors varying in age from 1 week to 29 months.

Materials and Methods.—Approximately 175 (C3H/Anf Cum ♀ × 101/Cum ♂) donors, ranging in age from 1 week to 29 months, and 300 twelve-week-old isologous recipients were used in this study. The mice were caged in groups of five and allowed free access to food and water. Within a day after a single total-body exposure to 800 r, the recipients were used as *in vivo* cultures for spleen cells. A G.E. Maxitron X-ray machine was used. Irradiation conditions were as follows: 300 kvp at 20 ma; 170 r/min at 70 cm; inherent filtration, 4.75 mm of Be; added filtration, 3 mm of Al; and hvl, 0.470 mm of Cu. The 30-day LD_{50} and LD_{99} doses for these 12-week-old mice are ~750 and ~875 r, respectively.[6]

Cell suspensions were prepared by teasing spleens of donors in Tyrode's solution containing 1 per cent normal isologous mouse serum. After determining the viability of spleen cells by the Eosin dye uptake method of Schrek,[7] the concentration of cells was adjusted appropriately and a 1-ml aliquot was then injected intravenously into each irradiated mouse. Intraperitoneal injection of the test antigen, 1 ml of 1 per cent sheep RBC, immediately followed this cell-infusion procedure, and individual serum samples were collected 6 days later. Sera were frozen until completion of the experiment and then titrated by our standard twofold serial dilution agglutinin method.[8]