Figure 1, A and B, clearly shows a decrease in the intensity of this peak, indicating that no significant increases in ADP occurred. If present, AMP can generally be well resolved at around +4 ppm, yet no major resonance was detected in this range. Concomitant with the loss of ATP, there was a dramatic increase in the intensity of the inorganic phosphate ($P_i$) peak, indicating that ATP was hydrolyzed and not simply sequestered or precipitated (*7*). These observations suggest that ATP is hydrolyzed to the free nucleoside, which is not observed by $^{31}P$ NMR.

Intracellular *p*H was also observed to collapse after heat shock (Fig. 1). The *p*H decreased less rapidly than the ATP concentration and reached its final value over a period of approximately 10 minutes. At an external *p*H of 6.2, the intracellular *p*H, which is usually about 7.2, shifted to approximately 6.7 upon heat shock ($N = 3$). When the external *p*H was adjusted to 7.2, the intra- and extracellular phosphate peaks coincided at +2.7 ppm. If there were intracellular acidification after heat shock and no decrease in internal $P_i$, we would expect to observe the appearance of an upfield resonance (at about +2.1 ppm). This did not occur, indicating that the intracellular medium was not acidified by more than 0.2 *p*H unit upon heat shock at the higher external *p*H. These results suggest that upon heat shock and loss of cellular ATP, cells become less able to maintain an alkaline *p*H gradient. They also suggest that lowered intracellular *p*H is not a condition for the heat shock response.

These observations indicate that the changes in cellular physiology associated with heat shock are more dramatic than was previously realized. We detected significant decreases in the steady-state levels of cellular ATP within 3 minutes after heat shock, which are well correlated with the existence and extent of heat shock protein synthesis. Like the transcriptional activation of the heat shock genes, these changes occur essentially immediately upon initiation of heat shock. It will be of interest to determine whether these changes in ATP are involved in triggering the general cellular response to heat shock.

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4. Cells were grown to mid-logarithmic phase ($2 \times 10^5$ cell/ml) in 2 percent proteose peptone, 0.1 percent yeast extract, 0.2 percent glucose, and 0.003 percent sequestrin at either 28°C (*T. pyriformis*) or 30°C (*T. thermophila*) and were harvested by gentle centrifugation. The pellets were washed and resuspended at $7 \times 10^6$ cell/ml in 20 m*M* 2-(*N*-morpholino)ethanesulfonate, 1.0 m*M* sodium phosphate, *p*H 6.0. This cell density represents an approximately 25 percent (by volume) cell suspension, which corresponds to an intracellular : extracellular volume ratio of 10 percent. Concentrated cultures (30 ml) were maintained for up to 6 hours with shaking at 250 rev/min in 250-ml culture flasks. At the beginning of each experiment, 14-ml samples were placed in 20-mm NMR tubes capped with an aeration manifold through which a mixture of 95 percent $O_2$ and 5 percent $CO_2$ was bubbled at a rate that maintains the $O_2$ tension well above the Michaelis constant $K_m$ for $O_2$ uptake (*8*). Samples were placed in a Bruker WH-360-WB NMR spectrometer operating at 145.78 MHz in pulsed Fourier-transform mode. Spectra were accumulated and stored approximately every 7.5 minutes as the sum of 300 free induction decays arising from 45° tipping pulses applied every 1.5 seconds. In some cases, different acquisition parameters were used to rule out possible effects of temperature on the spin-lattice relaxation time ($T_1$) that could lead to erroneous concentration values. The ATP levels were quantified by comparison with the known external phosphate concentration, correcting for both saturation differences and the intracellular : extracellular volume ratio. The *p*H was calculated from the chemical shifts of the $P_i$ peaks (*8*).

   After 1 hour of spectrum accumulation at standard temperatures, samples were withdrawn from the magnet and 0.4 ml was removed for protein labeling. The NMR sample was then brought to 33°C (*T. pyriformis*) or 40°C (*T. thermophila*) within 30 seconds by briefly immersing the spectrometer tube in a water bath at 55°C and then in a second bath equilibrated at the final heat shock temperature. During this time, the temperature of the NMR probe was also increased to the heat shock temperature. Samples were returned to the probe within 2 minutes of the heat shock. After one spectrum was acquired at the higher temperature (5 minutes), the sample was again withdrawn from the magnet, the temperature checked, and a second 0.4-ml portion removed for protein labeling. The sample was then returned to the magnet to continue spectrum accumulation.

   Portions removed for labeling of proteins were incubated with oxygenation at the control or the heat shock temperature. These portions contained $2.5 \times 10^6$ cells and were labeled in the presence of 75 μCi/ml of high specific activity $^3H$-labeled amino acid mixture (Amersham) for 1 hour. Whole cell protein was prepared as described in (*2*), except that 8*M* urea was added to the lysis buffer, which was heated to 100°C. Equal volumes of lysate from approximately $1 \times 10^5$ cells were analyzed on a 10-cm, 10 percent SDS acrylamide gel (*9*), and were then processed for fluorography (*10*).
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7 October 1982

## Infection of Normal Human Epithelial Cells by Epstein-Barr Virus

Abstract. *Primary cultures of epithelial cells were grown from the tonsils and adenoids of patients with diseases not related to Epstein-Barr virus. The cells could not be infected by Epstein-Barr virus. Fluorescein-labeled Epstein-Barr virus and a cytofluorograph were then used to show that the epithelial cells do not have detectable receptors for the virus. However, implantation with Epstein-Barr virus receptors gave the cells the ability to bind the labeled virus. One to 5 percent of receptor-implanted cells exposed to the transforming B95-8 substrain of the virus expressed Epstein-Barr nuclear antigen. The early and viral capsid Epstein-Barr virus–determined antigens were not detected in the virus-infected cultures. The results show that normal human epithelial cells from the nasopharynx become susceptible to infection by Epstein-Barr virus when the membrane barrier resulting from the lack of viral receptors is overcome by receptor implantation.*

Nasopharyngeal carcinoma occurs mainly among Cantonese Chinese, Alaskan natives, and in people in some regions of northern and equatorial Africa. The disease is common among adults and can occur in children and adolescents as well (*1*). The undifferentiated histophathologic type of nasopharyngeal carcinoma is consistently associated with Epstein-Barr virus (EBV), a lymphotropic human herpesvirus that causes infectious mononucleosis and is implicated in the genesis of Burkitt's lymphoma (*2*). Epstein-Barr virus DNA and Epstein-Barr nuclear antigen (EBNA) can be demonstrated in biopsy specimens of nasopharyngeal carcinoma (*3*). Epithelial cells, but not the lymphocytes

infiltrating the tumor, contain EBV genomes (*3*). Elevated titers of antibodies to viral capsid and early antigens are observed in blood and saliva of nasopharyngeal carcinoma patients, although the early antigens have not been found in the carcinoma cells themselves (*3*).

In spite of these findings, attempts to infect normal human epithelial cells with EBV have been unsuccessful (*4*). The resistance of epithelial cells to EBV infection in vitro may be related to the absence of EBV receptors. Only human and certain nonhuman primate B lymphocytes have receptors for the virus and can be infected in vitro (*5*). We recently developed two techniques that allow infection of cells lacking EBV receptors (*6*). EBV can be co-reconstituted with Sendai virus envelopes and microinjected into the cells during evelope-cell fusion. Alternatively, EBV receptors can be co-reconstituted with Sendai virus envelopes and transplanted onto the cells, which are then exposed to intact virus (*6*).

Using the latter technique, we have succeeded in infecting human T-cell lymphoma lines Molt-4 and 1301 (*7*), mouse lymphocytes (*8*), and 20 other cell lines of various origins, including human epithelial cells from pulmonary carcinoma. We now report that normal human epithelial cells do not have EBV receptors and cannot be infected by EBV. However, after being implanted with EBV receptors, the cells can be infected and express EBNA.

Primary cultures of human nasopharyngeal epithelium were grown from explants of tonsils and adenoids obtained from young children. Migration of epithelial cells from specimens began 4 to 5 days after explantation. These cells grew as monolayers and had the typical morphology of epithelial cells (Fig. 1). Electron microscopic observations of thin sections of the cells revealed desmosomes. Growth of fibroblasts from some explants was also observed; these cultures were discarded. Several attempts to infect the explanted human epithelial cells with EBV were unsuccessful, as judged by the lack of expression of EBV-specific antigens. To determine whether the latter resulted from nonsusceptibility of epithelial cells to EBV, we tested the cells for the presence of EBV receptors by using fluorescein isothiocyanate (FITC)–labeled EBV and an Ortho cyto-

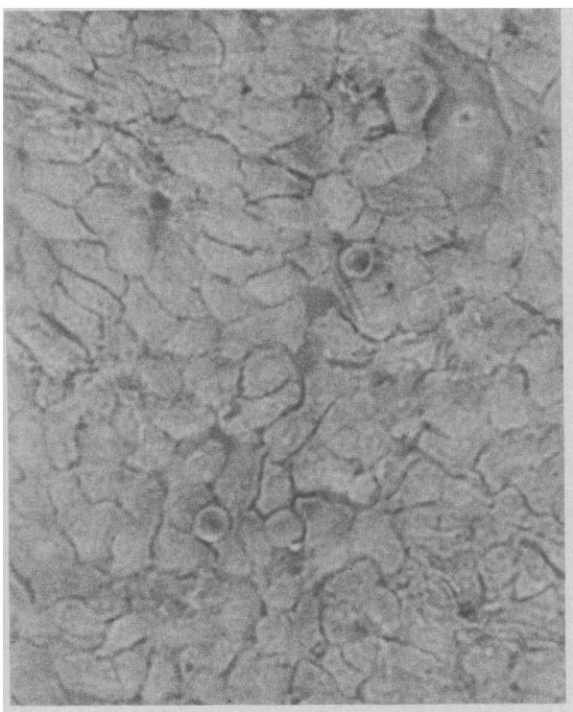

Fig. 1. Monolayer of human epithelial cells grown in a primary culture for 2 weeks after explantation of tonsils. Tonsils removed by surgery were washed in phosphate-buffered saline (PBS) supplemented with penicillin (500 U/ml), streptomycin (0.5 mg/ml), and gentamycin (0.5 mg/ml). Pieces measuring 1 to 2 mm were explanted in plastic petri dishes (Falcon) treated with fetal calf serum. Cultures were grown in RPMI-1640 medium supplemented with glutamine, 10 to 15 percent fetal calf serum, and antibiotics. (×150)

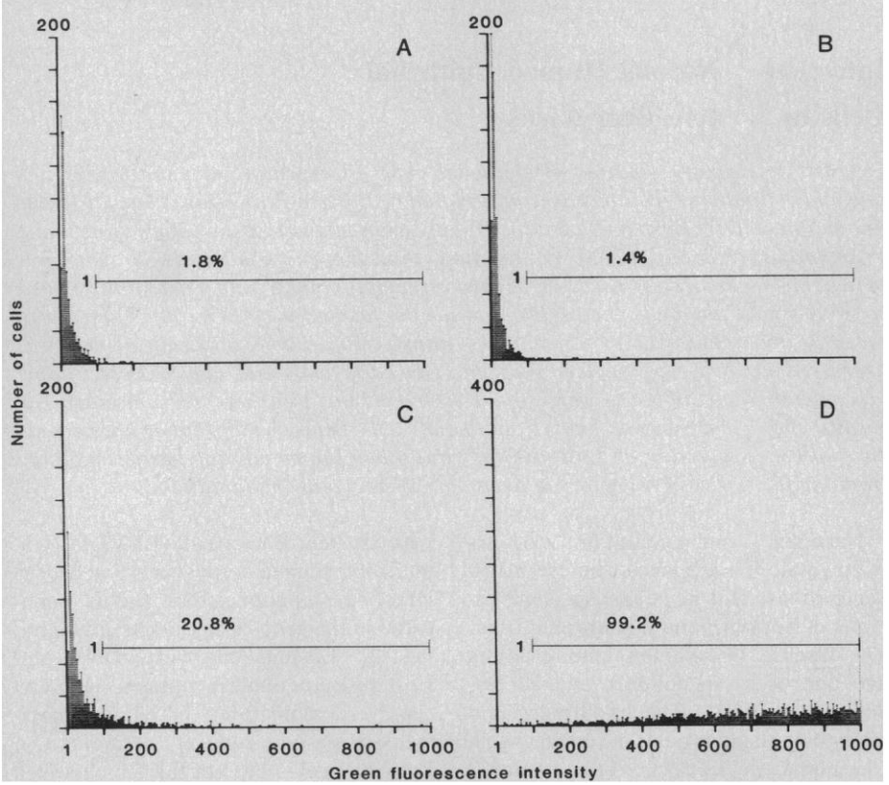


Fig. 2. Detection of EBV receptors on human nasopharyngeal epithelial cells by FITC-labeled EBV and a cytofluorograph. The B95-8 substrain of EBV, obtained from supernatant fractions of B95-8 cells, was concentrated 500 times and labeled with FITC. The labeled EBV was biologically active, as judged by its ability to induce EBNA in Ramos cells. Monolayers of epithelial cells grown from explants of tonsils were washed twice with PBS. Labeled EBV (50 μl) mixed with 250 μl of RPMI-1640 medium was added to a monolayer in each petri dish. After incubation for 1 hour at 4°C in darkness, the cells were washed two times, detached, and resuspended in PBS. (A) Control culture. Only RPMI medium was added to cell monolayers. (B) Adsorption of labeled EBV to intact epithelial cells. (C) Adsorption of labeled EBV to epithelial cells after EBV receptor transplantation. Lukes cells were used as a source of membranes containing EBV receptors. Co-reconstitution of the cell membranes and Sendai virus envelopes and EBV receptor transplantation by fusion of reconstituted membrane vesicles with target cells is described in (*6*). (D) Adsorption of EBV to Lukes cells ($2.5 \times 10^5$) incubated in 0.5 ml of RPMI medium containing 50 μl of labeled EBV.

fluorograph (*9*, *10*). The assay allows for receptor analysis at the level of a single cell, which is essential due to the difficulty of obtaining large numbers of single epithelial cells in suspension.

Normal human epithelial cells did not bind FITC-labeled EBV. The intensity of cell autofluorescence before addition of the virus (Fig. 2A) was identical to that observed afterward (Fig. 2B). Similar findings were obtained with other cells lacking EBV receptors, such as the human T cell, lymphoma cell line 1301, and mouse lymphoma cell line YAC-1. In contrast, nearly 100 percent of a B cell line derived from American Burkitt's lymphoma (Lukes; E. Kieff, University of Chicago) adsorbed labeled EBV (Fig. 2D).

It may be concluded that intact epithelial cells from the human nasopharynx do not have receptors for EBV. This finding is consonant with the recent unsuccessful attempts by Glaser *et al.* (*4*) to infect nonmalignant epithelial cells from the human nasopharynx, oropharynx, and nasal cavity with EBV (*4*).

The epithelial cells were then implanted with functional EBV receptors (*6*). About 20 percent of these cells acquired the ability to bind FITC-labeled EBV (Fig. 2C). Evidently, EBV receptor transplantation converted receptor-negative nasopharyngeal epithelial cells into receptor-positive ones. To examine the possibility that the receptor-implanted cells became susceptible to EBV infection, we exposed them to the transforming B95-8 substrain of EBV and stained for EBV-determined antigens 2 to 5 days later. We found in four separate experiments that 1 to 5 percent of EBV-infected epithelial cells contained EBNA (Fig. 3). Epithelial cells exposed to EBV without receptor implantation and cells not exposed to the virus were negative for EBNA when tested with serum samples containing antibody to EBNA. The proportion of EBNA-positive cells increased with the incubation time. Usually, 1 to 2 percent of cells were EBNA-positive when tested 2 days after infection. In one experiment, the number of EBNA-positive cells increased to 5 percent 1 week after infection. We did not succeed, however, in obtaining a fully EBV-positive culture of human epithelial cells.

No EBV-infected epithelial cells contained EBV-determined antigens characteristic for the virus-producing cycle. The cells were assayed by direct fluorescence with F1-Esther conjugate (for detection of early antigens) and F1-Buya conjugate (for detection of viral capsid antigens) prepared from the serum of patients with Burkitt's lymphoma. EBV-superinfected Raji cells and P3HR-1 cells (EBV-producer line of Burkitt's lymphoma origin) served as positive controls. While EBV-infected epithelial cells were negative for early and viral capsid antigens when stained with either conjugate 1, 2, or 5 days after infection, 15 to 20 percent of the superinfected Raji cells were positive for early antigen and 7 to 10 percent of P3HR-1 cells were positive for viral capsid antigen.

Recently, Stoerker *et al.* (*11*) demonstrated that tranfection of a human squamous cell carcinoma line of the nasal septum and a nasopharyngeal carcinoma line with purified DNA from the transforming or nontransforming substrains of EBV induces the formation of early antigen. Viral capsid antigen was detected after transfection of these cells with DNA from the nontransforming P3HR-1 substrain of EBV. Differences between our results and those of Stoerker *et al.* may be explained by the control of EBV expression exerted by the host cell. For example, the transforming B95-8 substrain of EBV induced only EBNA in human B lymphocytes and the human T-cell lymphoma line Molt-4, whereas it induced a viral lytic cycle in mouse lymphocytes (*7*, *8*). Transfection of human amniotic cells with DNA of EBV-infected B95-8 cells induced the viral lytic cycle (*12*). Microinjected DNA from EBV-infected P3HR-1 cells induced only early antigen in human and rat fibroblasts (*13*).

It remains unclear how EBV penetrates normal epithelial cells of the nasopharynx, which, as we have shown, lack EBV receptors. One possibility is fusion of the epithelium with EBV-infected lymphocytes. Cell fusion may be mediated by fusogenic proteins produced in EBV producer cells such as P3HR-1 (*14*).

Whatever the actual pathway of virus penetration, our results show that the expression of EBV DNA which occurs after virus enters normal epithelial cells is similar to that observed in human B lymphocytes, that is, it results in an early and exclusive induction of EBNA. Early and viral capsid antigens—EBV-determined antigens that are characteristic of

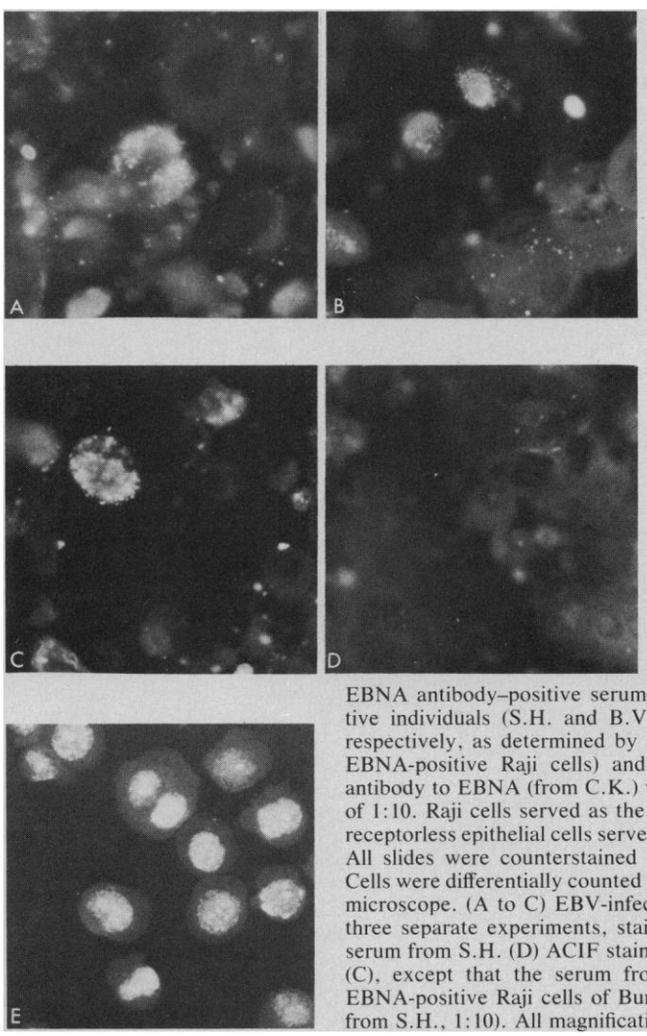


Fig. 3. Human epithelial cells stained for EBNA 5 days after infection by EBV strain B95-8. The cells (in monolayers) were implanted with EBV receptors and washed twice. One milliliter of EBV (concentrated 100 times) was added per petri dish and the cultures were incubated at 37°C for 1 hour, washed, and grown in RPMI-1640 medium. Five days after infection the cells were detached, washed twice in PBS, fixed in methanol and acetone (2:1) at −20°C for 5 minutes, and stained for EBNA by the anti-complement immunofluorescence (ACIF) test (*15*). In each test, one or two samples of EBNA antibody–positive serum from healthy, seropositive individuals (S.H. and B.V., titers 1:40 and 1:80, respectively, as determined by an end-point dilution on EBNA-positive Raji cells) and of serum negative for antibody to EBNA (from C.K.) were used at the dilution of 1:10. Raji cells served as the positive control and the receptorless epithelial cells served as the negative control. All slides were counterstained with Evans blue (1:10). Cells were differentially counted with a Zeiss fluorescence microscope. (A to C) EBV-infected epithelial cells from three separate experiments, stained for EBNA with the serum from S.H. (D) ACIF staining of the cells shown in (C), except that the serum from C.K. was used. (E) EBNA-positive Raji cells of Burkitt's lymphoma (serum from S.H., 1:10). All magnifications are ×160.

the viral lytic cycle—were not observed. It seems therefore that fresh human epithelial cells may, like human B lymphocytes, have control mechanisms that suppress the EBV lytic cycle. This suppression, concomitant with the expression of EBNA and induction of cellular DNA synthesis, is a prerequisite for the transformation of human B cells by EBV (*1, 3*). EBNA is observed in 95 percent of Burkitt's lymphona cases and in virtually all cases of undifferentiated nasopharyngeal carcinoma (*1, 3*). Thus the detection of EBNA in EBV-infected normal human epithelial cells strengthens the case for a role of EBV in the etiology of nasopharyngeal carcinoma.

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22 July 1982; revised 21 September 1982

## Stable Antibody-Producing Murine Hybridomas

Abstract. *A method is described for obtaining antibody-producing hybridomas that are preferentially retained in cultures of fused mouse spleen and myeloma cells. Hybridomas are produced by fusing mouse myeloma cells that are deficient in adenosine phosphoribosyltransferase (APRT) with mouse spleen cells containing Robertsonian 8.12 translocation chromosomes. The cell fusion mixtures are exposed to a culture medium that can be utilized only by APRT-positive cells, which results in the elimination of both unfused APRT-deficient myeloma cells and non-antibody–producing APRT-deficient hybridomas that arise by segregation of the 8.12 translocation chromosomes containing the APRT genes and the active heavy chain immunoglobulin gene.*

Cell fusion mixtures containing hybrids of mouse spleen and myeloma cells (hybridomas) are cultured in HAT medium (hypoxanthine, aminopterin, and thymidine) for 10 to 14 days to eliminate unfused hypoxanthine phosphoribosyltransferase (HPRT)–deficient myeloma cells (*1–9*). This selection system, however, can eliminate hybridomas that segregate the spleen X chromosome encoding the active HPRT locus (*9, 10*). For $HPRT^+$ antibody-producing hybridomas to survive in HAT medium they must retain the spleen X chromosome that encodes the active HPRT locus and the specific spleen autosomes that contain the active structural immunoglobulin (Ig) chain loci (chromosome 6, light chain kappa; or chromosome 16, light chain lambda; and chromosome 12, heavy chain) (*11, 12*).

One of us (R.T.T.) conceived a hybridoma selection strategy that permits ascertainment of the contribution of X chromosome segregation toward loss of hybridomas (Fig. 1) (*13*). In the Robertsonian (8.12) 5Bnr mouse [Rb(8.12) mouse] (*14*), the active heavy chain Ig locus on chromosome 12 (only one allele of the diploid complement is expressed) and a selectable enzyme marker locus (adenosine phosphoribosyltransferase, APRT) on chromosome 8 (*15, 16*) are genetically linked. Thus, the exposure of cell fusion mixtures to a medium requiring APRT activity ($APRT^+$ selection) eliminates both unfused $APRT^-$ myeloma cells and $APRT^-$ hybridomas. The latter are incapable of heavy chain Ig synthesis because of segregation of the 8.12 translocation chromosomes. For antibody-producing hybridomas to survive this selection procedure they must retain only the spleen chromosomes that contain the structural Ig chain loci. In this report we describe the production of hybridomas secreting antibodies for human pepsinogen I, and present evidence that during selection in HAT medium X chromosome segrega-

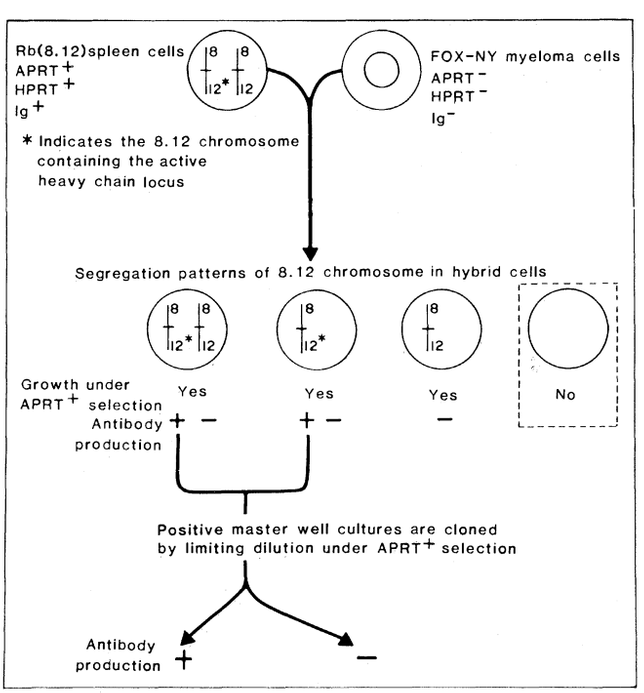


Fig. 1. Hybridization of Rb(8.12) spleen cells with FOX-NY myeloma cells and isolation of antibody-producing hybridomas under $APRT^+$ selection. The $APRT^+$ selection media (see text) contained azaserine to block de novo purine synthesis or aminopterin to block de novo purine and pyrimidine synthesis [for a review, see (*16*)]. Hybridomas that retain one or both 8.12 translocation chromosomes also produce APRT enzyme activity, which is necessary for survival under $APRT^+$ selection. Hybridomas that lose both 8.12 chromosomes as well as unfused $APRT^-$ myeloma cells are unable to utilize exogenous adenine to synthesize purines; exposure to $APRT^+$ selection medium eliminates them within 36 hours.