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The Spontaneous and Induced Synthesis of Epstein–Barr Virus Antigens in Raji Cells Immobilized on Surfaces Coated with Anti-lymphocyte Globulin

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SUMMARY

Immobilization of Raji cells on surfaces coated with anti-lymphocyte globulin (ALG) at low cell densities lead to the synthesis of Epstein–Barr virus (EBV) early antigen (EA) in up to 5% of the cells. At higher cell densities the percentage of antigen-positive cells decreased and at confluency no antigen synthesis was observed. Addition of iododeoxyuridine (IdUrd) to low density cultures increased the expression of EA to 20%, whereas in confluent cultures the cells could not be induced to synthesize EA. Treatment of cells in suspension with ALG failed to induce EA synthesis and did not potentiate the effect of IdUrd. Immobilized Raji cells proved to be suitable targets for superinfection with EBV derived from P3HR1 cultures.

Many experimental procedures which can be carried out with monolayers of cells cannot be adapted for experiments with cells which normally grow in suspension. Some examples are microinjection, titration of lymphotropic herpesviruses by plaque assay or investigation of the social behaviour of infected cells. Other procedures, such as pulse–chase experiments with lymphoblastoid cells, have presented problems since washing procedures often take 30 to 60 min and it is difficult to ensure that the cells are metabolically inactive during washing. Superinfected Raji cells are also extremely fragile and at late times after infection tend to disintegrate if subject to centrifugation. For these reasons we have developed a technique for the preparation of stable, viable, closely packed monolayers of lymphoblastoid cells (Bayliss & Wolf, 1980). Since we used anti-lymphocyte globulin (ALG)-coated surfaces to immobilize the cells it is necessary to investigate the expression of viral antigens within these cells as it has been shown (Tovey *et al.*, 1978) that treatment of Raji cells with antiserum to IgM induces the synthesis of the EBV early antigen (EA) complex. Since IgM is expressed on the surface of the Raji cell it was of interest to study the effect of an antiserum containing antibodies against a mixture of membrane components on the expression of EA within treated Raji cells and to compare the effects of ALG treatment in suspension and after fixation to a solid support.

Cultures of rapidly growing Raji cells were adjusted to contain 5×10^5 cells/ml and were dispensed into untreated Petri plates. ALG was added to the cultures at various concentrations (10^{-2} to 10^{-6} dilution of the stock ALG solution). Samples of the cultures were removed at 12, 24, 48, 72 and 96 h after addition of the ALG, and smears were prepared and stained for EBV-specified EA. No EA induction was observed in these cultures. At high concentrations of ALG the cells grew slowly and the cultures tended to have lower viabilities, while low concentrations of ALG which did not affect the viability of cells seemed to stimulate cell growth. Treatment of cells with IdUrd is known to induce EA expression (Hampar *et al.*, 1976). Therefore, we tested the ability of ALG-treated Raji cells to enter into EA synthesis after IdUrd treatment. At lower concentrations ALG treatment does not seem to affect the ability of the cells to produce EA. It is possible that higher ALG concentrations have an effect but the cells in these cultures were so badly clumped that it was not possible to count, with any accuracy, the numbers of positive and negative cells.

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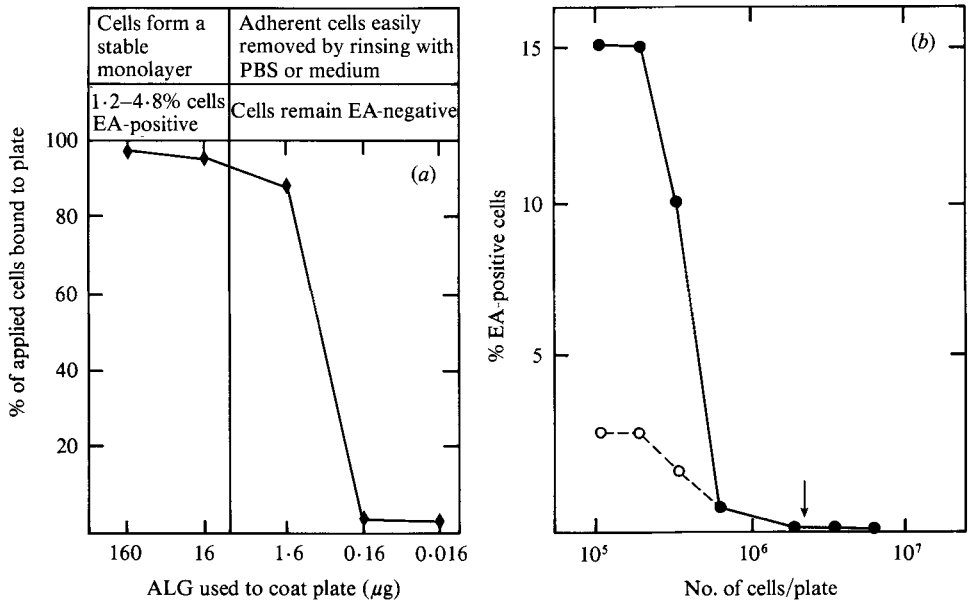

Fig. 1. Effect of ALG concentration on the attachment of Raji cells to plastic surfaces and the synthesis of EA in immobilized cells. 35 mm Petri plates were incubated with 2·5% glutaraldehyde at room temperature for 2 h and then washed with phosphate-buffered saline (PBS), 1 ml diluted ALG was applied to the plates which were incubated overnight and then washed to remove unadsorbed ALG. Cells at the required density were added and after 2 h the unattached cells were removed by rinsing the dishes with PBS, refed and incubated as indicated below. The monolayers were then rinsed with PBS, dried under a stream of cold air and fixed at −20 °C with petroleum–benzene (boiling point 50 to 75 °C) which preserved both the R and D components of the EA complex without attacking the plastic substratum. The fixed smears were stained directly using human serum conjugated with FITC or indirectly (Henle *et al.*, 1971) using human serum followed by FITC-conjugated anti-human IgG. The stained cells were embedded in semi-permanent medium (Rodriguez & Deinhardt, 1960) and examined with a Zeiss epi-illuminated fluorescence microscope. (*a*) Concentration of ALG necessary to obtain stable monolayers and spontaneous synthesis of EA (24 h after immobilization) within those monolayers. The range of values given is that observed in six experiments. (*b*) Effect of cell density on the spontaneous and induced synthesis of EA in Raji cells immobilized on Petri plates coated with ALG. The cells were immobilized at different densities, replicate cultures were treated with IdUrd (20 μg/ml) and 24 h after immobilization the cultures were stained for EA. ●, With and ○, without IdUrd. Arrow indicates position of cell confluency.

In a second series of experiments we studied the effects of ALG on Raji cells which were immobilized in Petri plates coated with ALG as described in the legend to Fig. 1. The data presented in Fig. 1 (*a*) indicate that a dilution of 10^{-2} of the stock ALG solution gave stable monolayers and that up to 4·8% of the bound cells spontaneously synthesized EA. At lower concentrations few cells were immobilized and most of the attached cells could be removed by gentle washing with PBS. Fig. 2 (*a*) illustrates Raji cells after incubation in an uncoated plate; cells are clumped, spherical and free floating. Fig. 2 (*b*) shows a culture of Raji cells immobilized on a partially coated plate; note the clear boundary between the coated and uncoated regions, the lack of cell clumps and that many cells have spread out and have an almost epithelioid morphology. In additional experiments we studied the synthesis of EA in immobilized cells as a function of cell density. Plates were coated with ALG as described and various numbers of cells were applied (between 10^5 and $6{\cdot}4 \times 10^6$ cells/plate). After the cells had adhered the cultures were incubated for 24 h and then stained for EBV EA as described. The results are presented in Fig. 1 (*b*). At low cell densities the cells spontaneously entered into EA synthesis, the number of EA-positive cells decreasing as the cell sheet approached

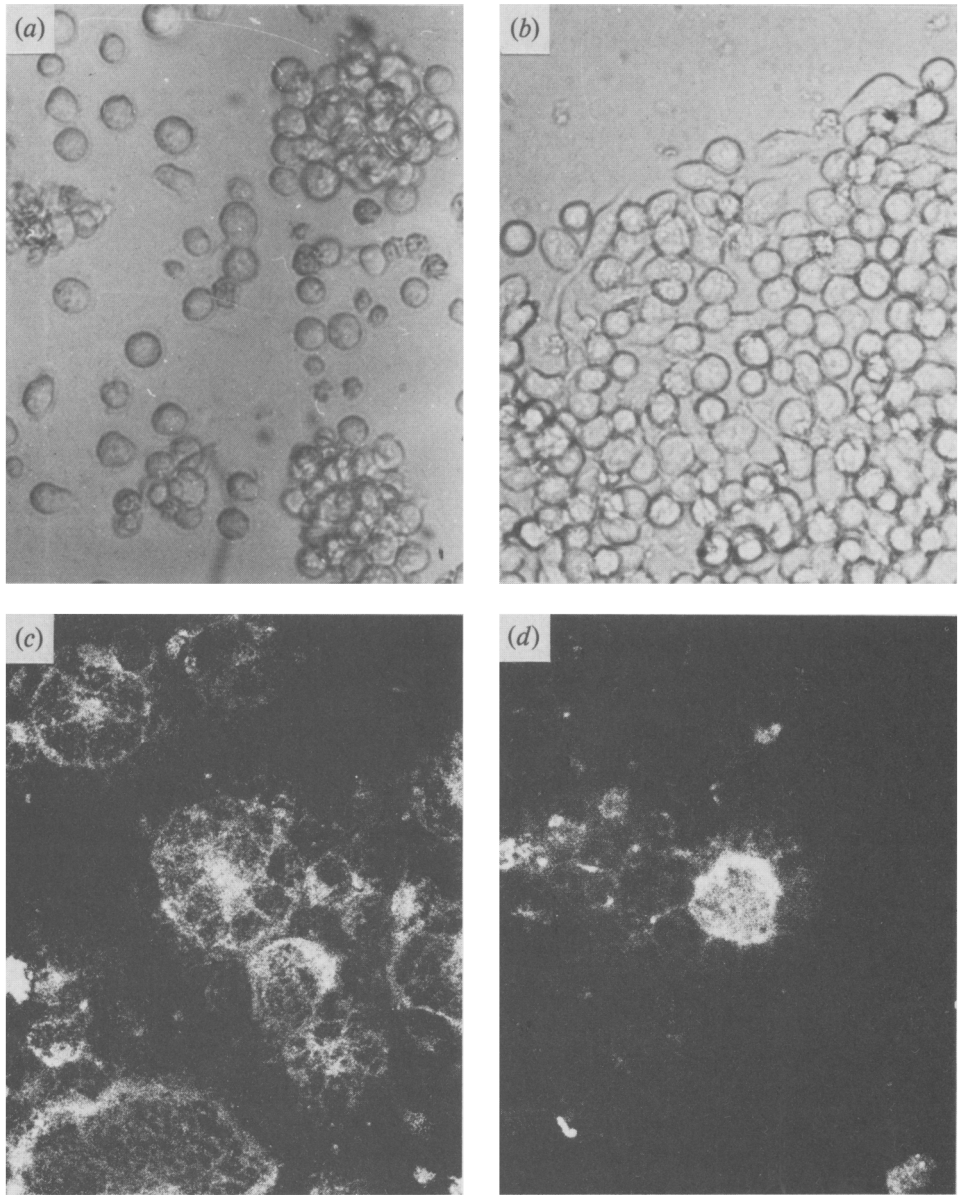

Fig. 2. (*a*) Raji cells in an uncoated Petri plate. (*b*) Raji cells in a Petri plate coated with ALG. (*c*) Immobilized Raji cells superinfected with concentrated virus; note partial lysis, fusion of cells and release of antigen. (*d*) As (*c*) but virus stock diluted 1:10; note apparent fusion of cells and spread of antigen to other cells. (*a*) and (*b*) are phase-contrast micrographs; (*c*) and (*d*) are photographs of fluorescent stains for EA (see Fig. 1 for methodology).

confluency, and in confluent monolayers no EA synthesis was observed. A series of experiments similar to those described above were carried out in the presence of IdUrd (20 μg/ml) which was added at the time of immobilization. In these experiments we observed that after treatment with IdUrd between 15 and 25% of the cells synthesized EA and that as the density of the cultures approached confluency their inducibility decreased (Fig. 1*b*). The fact that no EA was observed in cells in confluent monolayers even after treatment with IdUrd

offered a great advantage in that the cells could be used to study the induction of viral antigens by microinjection of purified viral DNA (Graessmann *et al.*, 1980; Wolf *et al.*, 1980*a*, *b*).

Since the above experiments showed that immobilized Raji cells were capable of EA synthesis, our next objective was to investigate their susceptibility to superinfection with EBV.

Raji cells were incubated with serial dilutions of an EBV stock derived from cultures of P3HR1 cells. Half of the infected cells were applied to ALG-coated plates and the remainder were cultured in suspension. All cells were incubated at 37 °C for 24 h. The monolayer cultures were rinsed with phosphate-buffered saline, (PBS) dried and then fixed with petroleum–benzene which preserves both the R and D components of the EA complex. Cells from suspension cultures were washed once with PBS and smears prepared as described by Henle & Henle (1966). The percentages of EA-positive and virus capsid antigen (VCA)-positive cells within the monolayer cultures were slightly higher than in suspension cultures. This may be explained by the sensitivity of superinfected Raji cells to centrifugal forces to which suspension cultures must be subjected during smear preparation. Fig. 2 (*c*, *d*) shows cultures stained with sera containing antibodies against EBV EA and VCA. At high concentrations of virus (Fig. 2*c*), almost all the cells are positive in the fluorescent antibody test and due to antigen leakage from the cells it is difficult to estimate the number of cells positive for the antigens. At lower dilutions the cells remain discrete and estimates of positive cells are more accurate. At the 1:10 dilution, foci of positive cells can be seen (Fig. 2*d*). This may be due to spread of the virus from an initially infected cell to surrounding cells since at the 1:100 dilution such spread is not observed. This observation correlates well with observations of Seigneurin *et al.* (1977) who demonstrated that productive infection of Raji cells with EBV is dependent upon the number of infecting EBV genomes. At low virus concentrations the cells required up to 72 h incubation before producing VCA, whereas concentrated virus stocks induced VCA synthesis within 7 h after superinfection. At higher m.o.i.s many polykaryocytes were observed in the cultures (Bayliss & Wolf, 1980).

The results of the experiments described above indicate that monolayers of Raji cells, prepared by the described technique, can be used for many experimental purposes. They provide a suitable milieu for virus replication, and studies published elsewhere (Graessmann *et al.*, 1980; Wolf *et al.*, 1980*a*, *b*) have shown that after polyethylene glycol-induced fusion, immobilized Raji cells provide suitable targets for microinjection of viral DNA. The dependence of polykaryocyte formation upon the multiplicity of infection also permits the titration of P3HR1-EBV preparations (Bayliss & Wolf, 1980).

The observation that immobilization of Raji cells at low cell densities induced the synthesis of viral antigen (EA) is an interesting finding, especially with respect to the data published by Tovey *et al.* (1978). They found that anti-IgM induced the synthesis of EA and that it potentiated the inducing properties of IdUrd. They also tested a number of other antisera directed towards membrane components (lipoprotein, β_2 microglobulin, C3 receptor, HLA histocompatibility antigens and Ia antigens) but found that only anti-IgM induced EA synthesis. The failure of the latter antisera to induce EA may have been due to low antibody titres or weak antigens. Alternatively, they may reflect some specificity in the activation of EBV. The ALG preparation used by us contained no detectable antibodies against human IgM (indirect haemagglutination test). It would therefore seem that the induction of EA in the immobilized cultures is not due to the anti-IgM effect. The failure of the cells to express EA when seeded at high cell densities may indicate that cell growth is required for activation of EBV. This conclusion is supported by the fact that treatment of Raji cells in S phase with IdUrd and other substances induced EA much more efficiently and after a shorter time than treatment of unsynchronized cells (Hampar *et al.*, 1976).

The failure of ALG to induce EA synthesis in suspension cultures may be explained by the inhibitory effect of the antiserum on cell growth, at high concentrations (10^{-2} and 10^{-3}

dilution). At lower concentrations the antiserum stimulated cell growth but may have been too dilute for EA induction.

Making the assumption that the *in vitro* effects of induction of viral genes by antibodies directed towards surface antigens of the EBV carrier cells also occur *in vivo*, one could speculate that the presence of autoantibodies in EBV-positive individuals could induce EBV-carrying cells to enter into a cycle of EBV expression which under normal circumstances remains latent within the B-lymphocytes.

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Max von Pettenkofer Institute
Pettenkoferstrasse 9a
8000 Munich 2, West Germany

GARY JAMES BAYLISS*
HANS WOLF

REFERENCES

BAYLISS, G. J. & WOLF, H. (1980). Epstein-Barr virus-induced cell fusion. *Nature, London* **287**, 164–165.

GRAESSMANN, A., WOLF, H. & BORNKAMM, G. W. (1980). Expression of Epstein-Barr virus genes in different cell types after microinjection of viral DNA. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 433–436.

HAMPAR, B., LENOIR, G., NONOYAMA, M., DERGE, J. G. & CHANG, S-Y. (1976). Cell cycle dependence for activation of Epstein-Barr virus by inhibitors of protein synthesis or medium deficient in arginine. *Virology* **69**, 660–668.

HENLE, G. & HENLE, W. (1966). Immunofluorescence in cells derived from Burkitts lymphoma. *Journal of Bacteriology* **91**, 1248–1256.

HENLE, G., HENLE W. & KLEIN, G. (1971). Demonstration of two distinct components in the early antigen complex of Epstein-Barr infected cells. *International Journal of Cancer* **8**, 272–282.

RODRIGUEZ, J. & DEINHARDT, F. (1960). Preparation of semipermanent mounting medium for fluorescent antibody studies. *Virology* **12**, 316–317.

RODT, H., THIERFELDER, S., THIEL, E., GÖTZE, D., NETZEL, B., HUHN, D. & EULITZ, M. (1975). Identification and quantitation of human T-cell antigen by antisera purified from antibodies crossreacting with hemopoietic progenitors and other blood cells. *Immunogenetics* **2**, 411–430.

SEIGNEURIN, J.-M., VUILLAUME, M., LENOIR, G. & DE THÉ, G. (1977). Replication of Epstein-Barr virus: ultra structural and immunofluorescent studies of P3HR1-superinfected Raji cells. *Journal of Virology* **24**, 836–845.

TOVEY, M. G., LENOIR, G. & BEGON-LOURS, J. (1978). Activation of latent Epstein-Barr virus by antibody to human IgM. *Nature, London* **276**, 270–272.

WOLF, H., BORNKAMM, G. W. & GRAESSMANN, A. (1980*a*). Expression of Epstein-Barr virus genes in different cell types after microinjection of viral DNA. In *Advances in Comparative Leukemia Research*, pp. 379–380. Edited by D. John, B. Lapin and J. Blakeslee, New York: Elsevier.

WOLF, H., BORNKAMM, G. W., GRAESSMANN, A., HUDEWENTZ, A. & BAYLISS, G. J. (1980*b*). Microinjection of Epstein-Barr viral DNA as a tool to study the expression of viral genes in monolayer cells and lymphoblastoid cells immobilized on solid supports. *Proceedings of the 9th International Symposium on Comparative Research on Leukemia and Related Diseases*, Moscow. Edited by B. A. Lapin and E. K. Dzhikidze (in press).

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