

Effect of Hydrocortisone on Cell Viability, Epstein-Barr Virus Genome Expression, and Interferon Synthesis in Human Lymphoblastoid Cell Lines¹

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SUMMARY

The effect of hydrocortisone on six human lymphoblastoid cell lines was measured in relation to cell growth, number of Epstein-Barr (EB) virus antigen-positive cells, number of EB virus particles, and interferon synthesis. In two of the six cell lines (HRIK, MCI), hydrocortisone, in proportion to the dose, adversely affected cell viability concomitantly enhancing the expression of the EB virus genome. Recovery of normal viability in these two cell lines or lack of effect in the other three cell lines (EB3, Roswell Park Memorial Institute 1788, and AGI) appeared to be related to interferon synthesis. The observed effects occurred only in cell lines (HRIK, MCI) showing no detectable interferon prior to hydrocortisone, and recovery in these cell lines coincided with the appearance of detectable interferon. Conversely, in the three cell lines where interferon was either already present (AGI) or appeared within 3 days (EB3, Roswell Park Memorial Institute 1788), no adverse effect on cell viability and no definite activation of the EB virus genome could be demonstrated. In Raji, a few antigen-positive cells (4 to 23 per 10⁵ cells) appeared but no viral particles. Cell viability was virtually unaltered and interferon was not detected. In Raji following bromodeoxyuridine (20 mg/ml) up to 1200 antigen-positive cells per 10⁵ cells appeared with an associated interferon response. Priming of the HRIK cells with performed human interferon, 500 to 1000 units/ml of cell suspension for 72 hr or more prevented the adverse effect of hydrocortisone (20 to 100 µg/ml) on cell viability. These findings may help to reestablish a relationship between interferon synthesis and viral replication in EB virus-carrying lymphoblastoid cell lines.

INTRODUCTION

The effect of hydrocortisone on the synthesis of interferon and on the activity of preformed interferon in relation to viral replication in various tissue culture systems has been investigated by several workers with variable results (5, 15, 17, 19, 21, 23, 24). Hydrocortisone has been shown to

adversely affect the viability of peripheral lymphocytes (9) but not of thymus cells (1) in short-term culture. Hydrocortisone has also been reported to decrease the viability of lymphoblastoid cells in certain murine lymphoma cell lines but not in others (3, 8, 11, 22). We could find no data, however, concerning the effect of hydrocortisone on cell viability, on the expression of the EB² virus genome, and on interferon synthesis in human lymphoblastoid cell lines. We therefore decided to study the effect of hydrocortisone on 6 human lymphoblastoid cell lines in relation to cell growth, number of EB virus antigen-positive cells, number of EB virus particles released, and interferon synthesis.

MATERIALS AND METHODS

The 6 human lymphoblastoid cell lines used in this experiment were HRIK, Raji, RPMI 1788, EB3, AGI, and MCI. Clone HRIK (kindly supplied by Dr. Werner Henle, The Children's Hospital, Philadelphia, Pa.) is a lymphoblastoid cell line originally derived from a Burkitt lymphoma. It actively synthesizes EB virus antigens and also produces EB virus particles. The Raji cell line, derived from a Burkitt lymphoma, carries the EB virus genome in a repressed state. The RPMI 1788 cell line, established from the blood of a healthy donor, is positive for the EB virus genome but synthesizes EB virus antigens and produces particles at a much lower rate than HRIK. The RPMI 1788 cell line was obtained from Associated Biomedic Systems, Buffalo, N. Y. The EB3 line (kindly supplied by Dr. Werner Henle) was established by Epstein and Barr from a Burkitt lymphoma. The AGI and MCI cell lines were established in our laboratory from the buffy-coat cells of a patient with infectious mononucleosis and from a healthy donor, respectively. The EB3, AGI, and MCI cell lines disclosed a few EB virus antigen-positive cells but complete EB virus particles could be detected in MCI only by negative staining of high-speed pellets following several cycles of freezing and thawing followed by differential centrifugation of 80 ml of a cell suspension containing 10⁶ cells/ml. The AGI and MCI cell lines were established according to the technique of Moore *et al.* (4, 20) from 10 to 20 ml of heparinized blood. All cell lines were maintained in RPMI Medium 1640

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² The abbreviations used are: EB, Epstein-Barr; RPMI, Roswell Park Memorial Institute; BUdR, bromodeoxyuridine.

containing 10% heat-inactivated fetal calf serum with added penicillin and streptomycin at 37°.

The cell cultures used in the experiments (test cultures and controls) were prepared from at least a 1-liter suspension of each cell line containing 9.0×10^5 cells/ml by distributing 100 ml in each of several 16-oz prescription bottles. Hydrocortisone (Solu-cortef, obtained from The Upjohn Co. of Canada, Don Mills, Ontario, Canada, Lot 25,934) was added to the medium of the test cultures in concentrations varying from 0.01 to 100 $\mu\text{g}/\text{ml}$. At least 3 control cultures and 3 cultures for each dilution of hydrocortisone were checked daily for cell count and cell viability by the trypan blue dye exclusion test. The number of EB virus antigen-positive cells was determined after the examination of 300 cells by indirect immunofluorescence (10) using a known early and viral capsid-positive antiserum and a negative control serum (12, 13). To minimize the effect of cell concentration on cell growth and viability, the cell concentration was readjusted to 9×10^5 cells/ml every 3 or 4 days in all cultures. The hydrocortisone concentration was similarly maintained constant throughout the experiment. After 1, 2, 3, 7, 8, 9, 10, 13, 15, and 17 days, smears were prepared for immunofluorescence. Samples taken at 3, 7, 10, 13, and 17 days for electron microscopy and interferon assay were successively frozen and thawed 3 times and centrifuged. The sediment from the original cell suspension was resuspended in a ratio of 20/1 in RPMI 1640 medium. An amount of 0.01 ml of the sample was placed on a carbon-coated grid resting on an agar plate. After a 90-min contact, the grid was dried completely with a filter paper and a 3% solution of phosphotungstic acid, pH 6.0, was added. The supernatant fluid was brought to pH 2 and left overnight, dialyzed against phosphate-buffered saline, concentrated 10-fold with Carbowax, dialyzed for 7 hr against Earle's minimal essential medium and centrifuged for 1.5 hr at $80,000 \times g$. It was subsequently assayed (2) in 2-fold dilutions for interferon activity on WI38 cells. The cell monolayer was washed following the 17-hr contact period with the interferon preparation. The vesicular stomatitis virus was used as challenge virus. The titers were obtained by graphical interpolation and represent the final dilutions of the interferon preparations at which there would have been 50% protection of the cell sheets. Dilutions of hydrocortisone in RPMI Medium 1640 were used as control. Exogenous human interferon of high titer was kindly supplied by Hans Stränder, Karolinska Institutet, Stockholm, Sweden. Prediluted 1000 times it gave a titer of 150/ml in the WI38-VSV test system compared to 362/ml for the WHO interferon standard of reference Lot No. 69/19 containing 5000 units/ml. Interferon was used for priming of the lymphoblastoid cell lines in doses of 500 to 1000 units/ml of cell suspension for periods of 1, 3, 72, and 144 hr prior to the addition of hydrocortisone.

RESULTS

Hydrocortisone, 0.01 $\mu\text{g}/\text{ml}$, was without effect. Hydrocortisone, 0.1 $\mu\text{g}/\text{ml}$ to 100 $\mu\text{g}/\text{ml}$, had an adverse effect

upon cell viability and upon the expression of the EB virus genome in 2 "responsive" cell lines (HRIK and MCI). Up to 43% of the MCI and 30% of the HRIK cells were nonviable by trypan blue dye exclusion test after 3 days of incubation compared to a maximum of 15 and 12%, respectively, for the control cells (Chart 1). Hydrocortisone had no such effect in the other 3 "unresponsive" cell lines (EB3, AGI, and RPMI 1788). In Raji no definite effect upon cell viability was observed following the addition of hydrocortisone. In the HRIK cell line with added hydrocortisone, a 2- to 3-fold increase in the number of EB virus particles compared to the control could be demonstrated with a maximum effect between Days 3 and 7 (Table 1; Chart 1). An earlier increase was observed in the MCI cell line maximum at 2 rather than 3 days. Appearance of EB virus antigen-positive cells or a significant increase in their number (from 0 to $4/10^5$ cells to 4 to $23/10^5$ cells) was observed in the Raji cell line but virus particles were not detected. The magnitude of the response was proportional to the dose in all 3 cell lines, HRIK (Chart 2), MCI, and Raji. The timing of the response likewise was related to the dose; it was earlier with 100 μg and later with 15 μg . Two additional experiments showed the same effect of hydrocortisone on cell viability, the number of EB virus antigen-positive cells, and the number of EB virus particles using MCI and HRIK cell lines. The effect of hydrocortisone was reproducible in the 2 successive experiments done with the Raji cell line. Under the effect of hydrocortisone, therefore, the percentage of antigen-positive cells increased from an average of 3.5 to an average of 9.7% in HRIK, whereas in Raji this percentage increased from less than 0.0013 to 0.0145% (over a 10-fold increase). Standard deviation remained within acceptable limits (Table 1). For comparative purposes, the average increase in antigen-positive cells noted in our laboratory in Raji following BUdR (20 $\mu\text{g}/\text{ml}$) went from 0.0013 to 0.12% (close to a 100-fold increase) (Chart 3).

The base-line level of interferon (or interferon-like activity) in the supernatant fluid of these cultures concentrated 10-fold remained undetectable except in the AGI cell line with an interferon titer of 8. Following the addition of hydrocortisone, interferon was easily detected in all cell lines at 3 days except in HRIK and Raji (Table 1). In the MCI cell line, however, the interferon titer of 4 at 3 days was lower than in the AGI, EB3, and RPMI 1788 cell lines (titer, 24, 20, and 20, respectively). In subsequent experiments in which the interferon response was measured not only after 3 days but after 7 and 10 days, a detectable interferon titer was finally obtained in HRIK (Table 1; Chart 1), but only after 10 days (after 7 days with the 100 μg dose), and the magnitude of the response was proportional to the dose of hydrocortisone (Chart 2). Recovery of cell viability equal to that of the control cells was achieved on Day 8 in the MCI cell line and after 2 to 3 weeks in the HRIK cell line following the interferon response in both cases. In the Raji cell line, the effect on the expression of the EB virus genome was maximum from the 7th to the 10th day but persisted to the end of the test period with no detectable interferon level in the supernatant fluid (Chart 3).

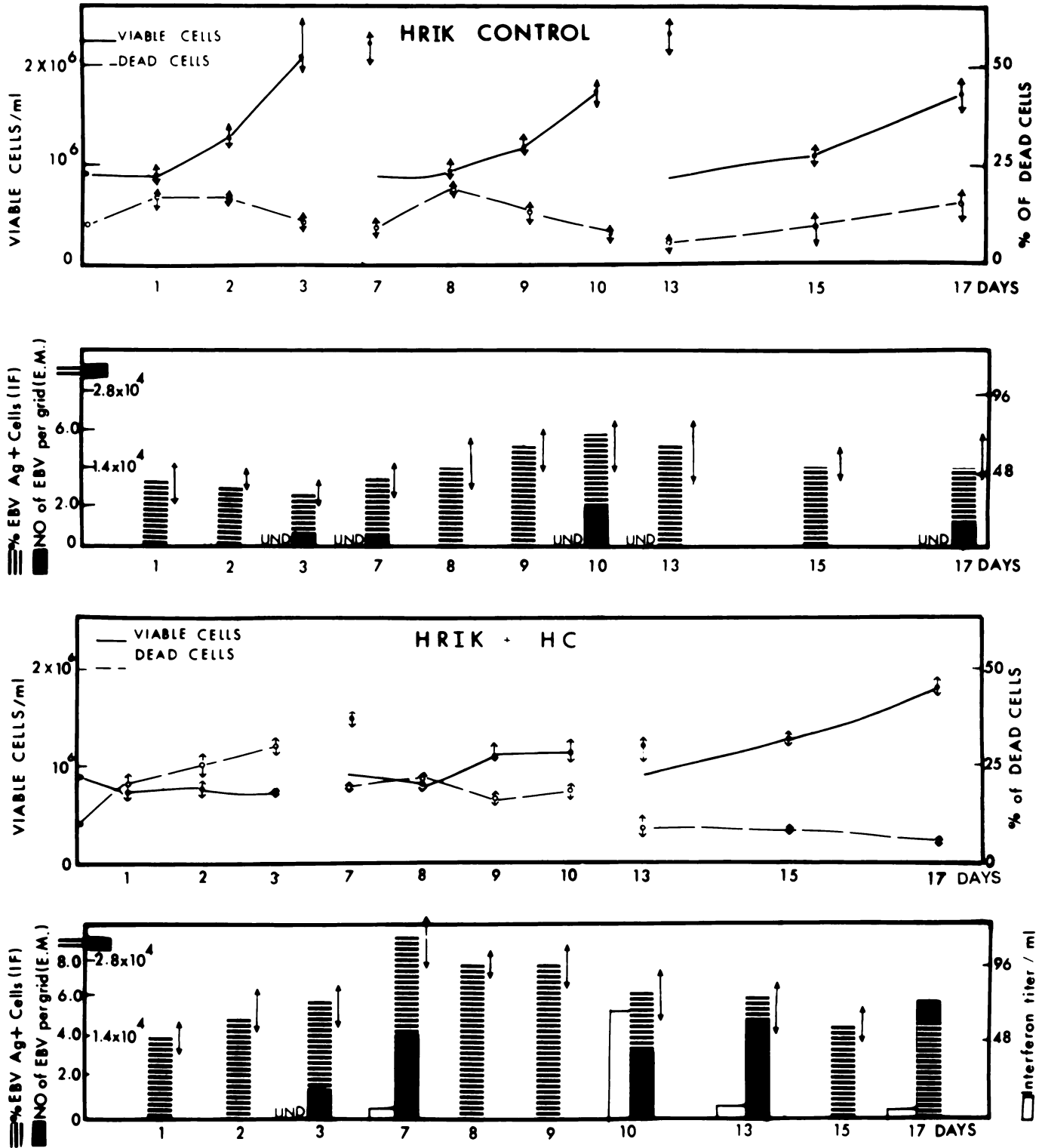


Chart 1. Effect of hydrocortisone (100 $\mu\text{g}/\text{ml}$) on cell viability, EB virus genome expression, and interferon titer in HRIK over a prolonged period. The interferon titer was obtained by graphical interpolation, and represents the final dilutions of the interferon preparations at which there would have been 50% protection of the cell sheets. On Days 3, 7, 10, and 13, the medium was changed and the viable cell count was readjusted (900,000 cells/ml). *EBV*, Epstein-Barr virus; *Ag +*, antigen positive; *IF*, immunofluorescence; *E.M.*, electron microscopy; *UND*, undetectable interferon level; *HC*, hydrocortisone.

Table 1
Effect of hydrocortisone, 100 µg/ml, in 2 responsive cell lines and in Raji

	Cell line	Day										
		1	2	3	7	8	9	10	13	15	17	
% of dead cells in HC-treated cells/control cells ^a	HRIK	1.1	1.9	2.8	3.2	2.0	1.5	2.0	2.3	1.0	0.4	
	MCI	1.1	1.5	2.6	4.9	1.3	1.1	1.1	1.5	0.4	0.4	
	Raji	1.0	1.1	1.7	0.8	0.8	0.7	1.1	0.5	0.8	0.8	
Ratio of % EB virus antigen-positive cells in HC-treated cells/control cells ^b	HRIK	1.2	1.6	2.4	$\frac{10.9 \pm 1.2}{4.6 \pm 0.6}$	2.8	$\frac{9.7 \pm 0.6}{3.5 \pm 0.3}$	2.0	1.5	1.1	1.1	1.1
	MCI	1.4	2.5	$\frac{6.6 \pm 0.4}{2.6 \pm 0.4}$	2.0	1.3	0.8	1.2	1.3	1.4	0.9	1.1
	Raji ^c	2.8	3.1	5.1	11.2	$\frac{0.0145}{0.0013}$	6.7	8.0	8.6	6.0	2.4	2.0
Ratio of EB virus particles in HC-treated cells/control cells	HRIK			2.2	7.1			2.0	4.1	4.8		
	MCI			2.4	1.0			1.0	1.0	1.0		
	Raji			UND	UND			UND	UND	UND		
Interferon titer	Control cells	HRIK			NEG	NEG		NEG	NEG	NEG		
		MCI			NEG	NEG		NEG	NEG	NEG		
		Raji			NEG	NEG		NEG	NEG	NEG		
	HC-treated cells	HRIK			NEG	4		64	NEG	NEG		
		MCI			4	80		96	16	20		
		Raji			NEG			NEG	NEG	NEG		

^a HC, hydrocortisone; UND, undetectable in HC-treated and control cells; NEG, negative.

^b Three hundred cells were counted except in Raji where the total number of antigen-positive cells on a smear made of 10⁶ cells were counted.

^c Raji range in control cells, 0 to 4 EB antigen-positive cells/10⁶ cells; range in HC-treated cells, 4 to 23 EB antigen positive cells/10⁶ cells.

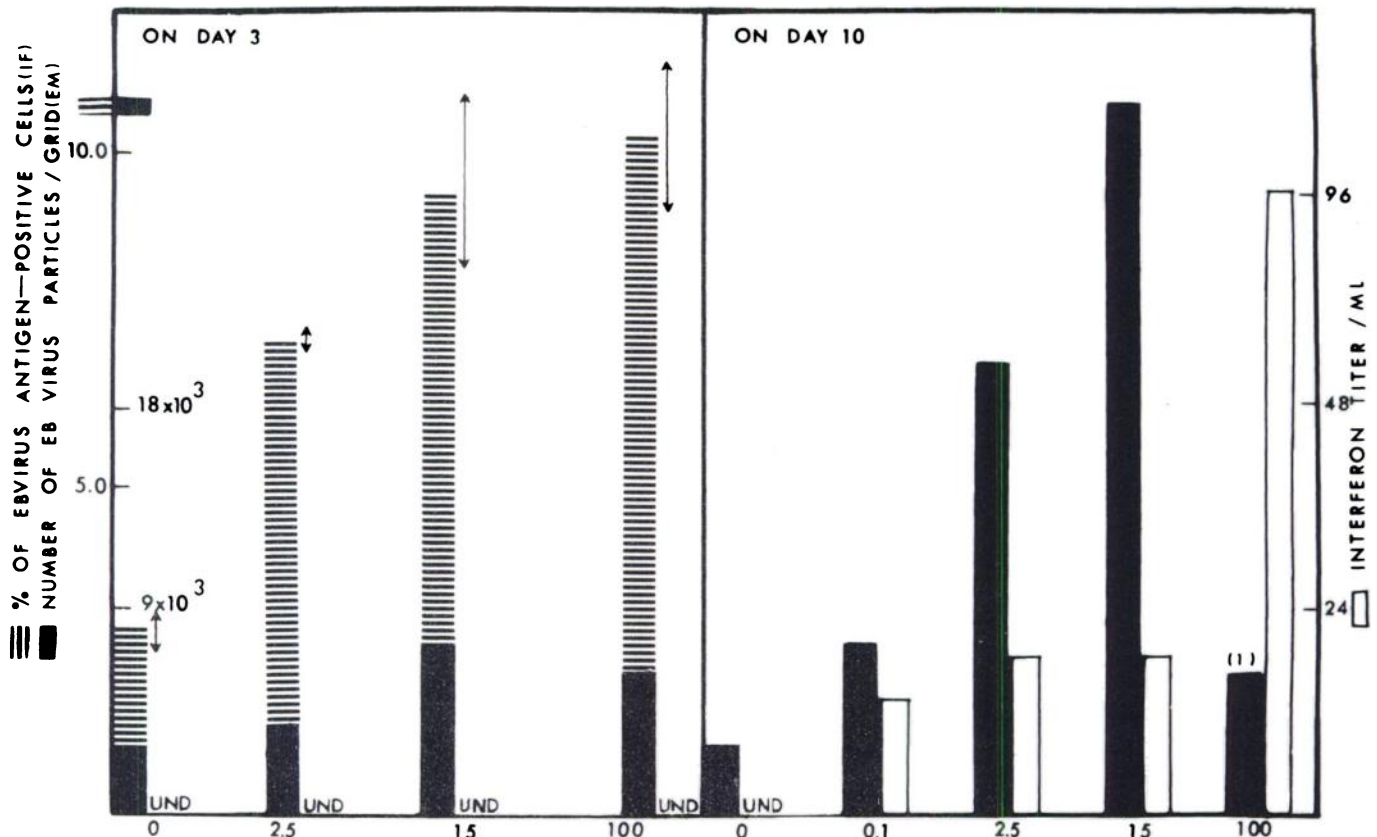


Chart 2. Effect of hydrocortisone (0.1, 2.5, 15, and 100 µg/ml) on the percentage of EB virus antigen-positive cells (IF), the number of EB virus particles (EM) and interferon titer in HRIK cells on Days 3 and 10. The effect of 100 µg of hydrocortisone per ml on the expression of the EB virus genome was maximum at 7 days. The WHO interferon standard (5000 U/ml; Lot No. 69/19) gave a titer of 362/ml in this assay. IF, immunofluorescence; E.M., electron microscopy; UND, undetectable interferon level.

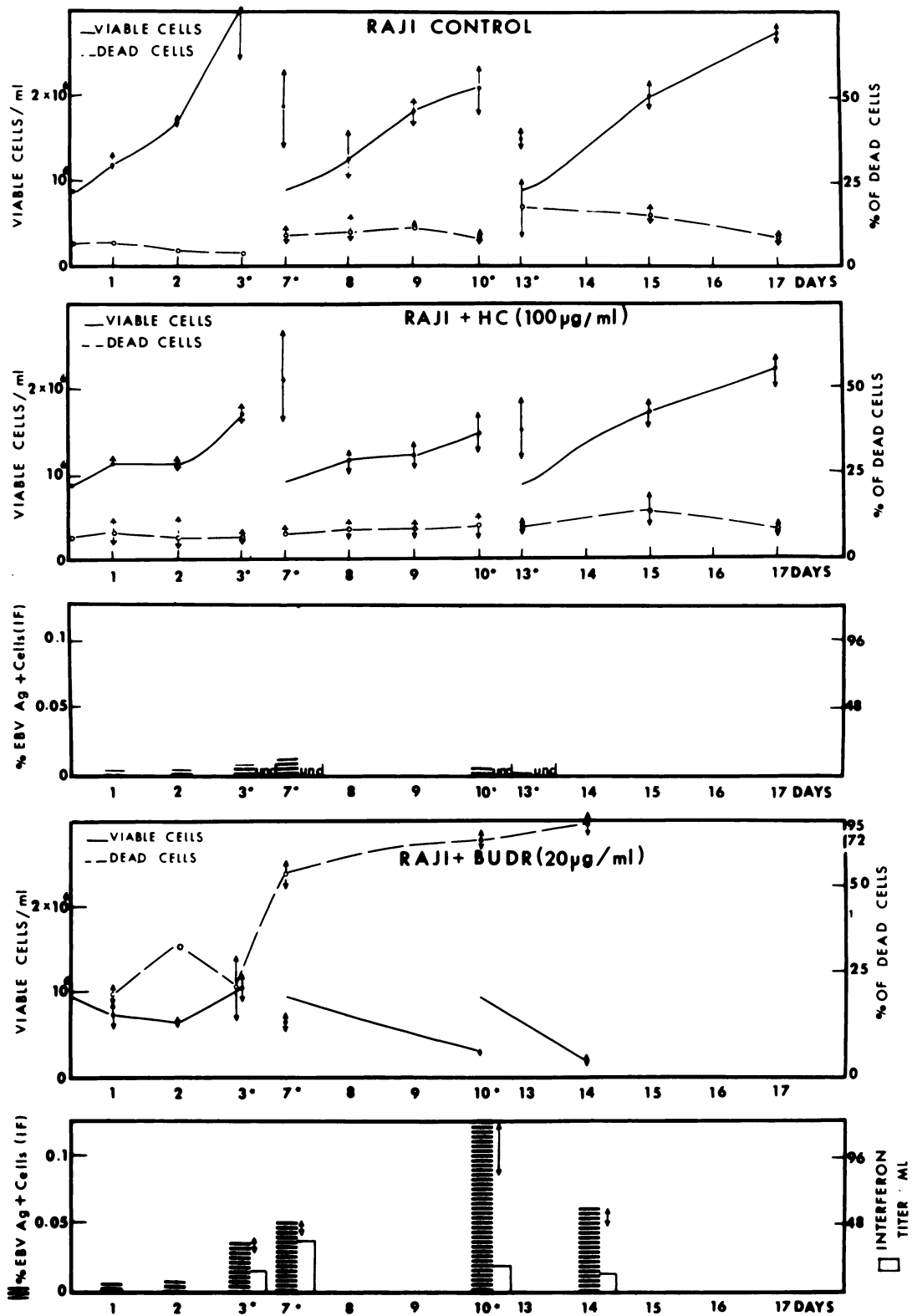


Chart 3. Effect of hydrocortisone (100 µg/ml) and BUdR (20 µg/ml) on cell viability, EB virus genome expression and interferon titer in Raji over a prolonged period. The interferon titer was obtained by graphical interpolation and represents the final dilutions of the interferon preparations at which there would have been 50% protection of the cell sheets. *, days on which the medium was changed and the viable cell count readjusted (900,000 cells/ml); HC, hydrocortisone; EBV, Epstein-Barr virus; Ag+, antigen positive; IF, immunofluorescence; und, undetectable interferon level; BUdR, BUdR.

Another experiment will have to be prolonged. Despite the continued presence of hydrocortisone, the observed effect appears to be transitory. Removal of hydrocortisone from the medium after 45 days in the HRIK or MCI cell line did not have any effect over a period of 15 days of observation. In Raji, following BUdR activation, an interferon titer of 16 was obtained after 3 days rising to 28 after 7 days and dropping to 6 after 14 days (Chart 3). Priming of the HRIK cells with preformed human interferon 500 to 1000 units/ml of cell suspension for 72 or 144 hr clearly prevented the effect of hydrocortisone (20 to 100 $\mu\text{g}/\text{ml}$) on cell viability but did not clearly prevent the observed effect on the EB virus genome expression. Hydrocortisone alone or added together with interferon without priming or following priming for less than 72 hr (3 hr and particularly 1 hr) produced the same adverse effect on cell viability as in previous experiments, whereas interferon alone (up to 1000 units/ml) was without effect on the HRIK cells.

DISCUSSION

There is evidence that, in certain murine lymphoma cell lines, hydrocortisone and other glucocorticoids adversely affect cell viability and inhibit glucose uptake and protein synthesis (8, 11, 22). The presence of cytoplasmic hormone receptors of proteinic nature seems to be a prerequisite for the sensitivity of the cell lines to the effect of the steroid. Hormone receptor-positive but resistant lines have been reported, however; therefore, the explanation for the sensitivity or resistance to hydrocortisone is not yet available (3). This phenomenon in murine lymphoma cell lines finally has not been studied in relation to viral replication or interferon synthesis (8, 11, 22).

If the primary effect of hydrocortisone in our experiments is on cell viability, secondarily affecting the expression of the EB virus genome, the observation that interferon prevents the adverse effect on cell viability is interesting and deserves further study in the light of current knowledge concerning the mechanism of action of glucocorticoids at the subcellular level (16). Both interferon and hydrocortisone may happen to interact at the translational level or may possibly compete for the same receptors.

If there is a direct effect of hydrocortisone on the expression of the virus genome, then exogenous interferon is only affording partial protection from this effect. In fact, large amounts of interferon for prolonged periods are required to influence the carrier state of other persistent tissue culture viral infections (7) in contrast to the limited amount needed to prevent exogenous virus infection. A possibility that would explain the results will be investigated: interferon could exert its control in cells not already engaged in a lytic cycle, mainly on the translation of early viral mRNA into proteins that play a part in the inhibition of cellular macromolecules synthesis. Hydrocortisone more complex in its action (16) could still influence, in spite of exogenous interferon, the transcription and translation of late viral (as well as early viral and cellular) messengers.

Whether the primary effect of hydrocortisone is on cell viability or on the expression of the virus genome, the

ensuing event is one that appears to be reactive interferon synthesis. A relationship between interferon synthesis and viral replication in EB virus-carrying lymphoblastoid cell lines has been denied by most investigators on the basis of available data relating the percentage of EB virus antigen-positive cells to interferon synthesis in "steady-state" conditions (6, 14, 18, 25). The observed enhancement induced by hydrocortisone in the expression of the EB virus genome and the concomitant altered cell viability followed by an increase in interferon synthesis with subsequent return to normal, may help to reestablish a kinetic relationship between interferon synthesis and EB virus replication. Base-line interferon levels found in this study are identical to those found by Haase *et al.* (6) in corresponding lymphoblastoid cell lines. The observation that either preexisting detectable interferon as in AGI or brisk interferon response as in RPMI 1788 and EB3 seems to prevent detectable activation of the EB virus genome and that, conversely, an undetectable interferon level prior to the experiment and a delayed interferon response as in HRIK result in activation of the EB virus genome following the addition of hydrocortisone is strong evidence in favor of a relationship between viral genome expression and interferon synthesis. That the magnitude of the response is dose related is further evidence for the specificity of the observed effect of hydrocortisone. The observations made in Raji following BUdR strengthen the postulated relationship between viral genome expression and interferon synthesis.

The mechanism by which hydrocortisone induces the observed changes either in murine lymphoma cell lines or in human lymphoblastoid cell lines is not clear. These preliminary findings raise the possibility that hormonal factors may also, *in vivo*, play an important part in the pathogenesis of disease states eventually linked to the EB virus (12). The observation that the most sluggish interferon response occurred in cell lines originating from Burkitt lymphomas and the quickest in a cell line derived from a subject with infectious mononucleosis should be investigated in a larger number of cell lines of diverse origin.

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