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Induction of Tubuloreticular Inclusions in Human Lymphoma Cells (Raji Line) Related to S-Phase Treatment with Halogenated Pyrimidines[1]

Barbara Hulanicka,[2] David W. Barry, and Philip M. Grimley[3]

Clinical Pathology Laboratories, Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201 [B. H., P. M. G.], and the Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20014 [D. W. B.]

SUMMARY

Formation of tubuloreticular inclusions (TRI) within the endoplasmic reticulum was initiated in human lymphoma cells (Raji line) by exposure of synchronized cultures to 5-bromo-2'-deoxyuridine (BUdR) for all or portions of a single S phase. The TRI were detected by electron microscopy as early as 36 hr after addition of 260 μM BUdR. By 72 hr, thin-section counts of TRI reached a maximum at circa 30% of cell cross-sections, which was estimated to represent no less than one TRI per two whole cells. Although the numbers of TRI depended upon the concentration of halogenated pyrimidine, cell division was not prerequisite to their appearance, and exposure of the cells to BUdR for repeated cycles of DNA synthesis did not increase the maximal level. These results were consistent with a unifilar dominance and modified regulation of gene expression, rather than transcription of an altered DNA. Raji cells exposed to equimolar thymidine as controls to each experiment, as well as untreated Raji cells, were uniformly negative for TRI. A relation of TRI induction to metabolism of BUdR was indicated by competitive inhibition with thymidine and lack of response in a subline selected for BUdR resistance. There was no evidence for previously postulated relationships of TRI to myxovirus infection or IgG production. *In vitro* induction of TRI can serve as an experimental model for further investigations into the significance of TRI found in lymphocytes of patients with systemic immune diseases or neoplasms.

INTRODUCTION

TRI[4] with distinctive ultrastructural and cytochemical features (16, 33) can occur spontaneously in human lymphoid cell lines established from a variety of clinical sources (8, 16). The frequency of such inclusions is typically low and inconstant (16). Some cell lines examined repeatedly by electron microscopy, including Raji cells of Burkitt's lymphoma origin, have proven consistently negative for TRI (13, 16). It was shown previously by Grimley *et al.* (13) that TRI can be induced to form in Raji cells during continuous treatment with BUdR, and this was confirmed with another Burkitt's lymphoma line (EB-3) examined by Splinter *et al.* (36). We now report more extensive experiments in which the specificity of TRI induction by halogenated pyrimidines has been investigated in synchronized Raji cell cultures.

MATERIALS AND METHODS

Cells. Raji cells originally provided through the courtesy of Hope Hopps, Bureau of Biologics, U. S. Food and Drug Administration, were propagated in spinner flasks at 37° in Roswell Park Memorial Institute 1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with glutamine (292 mg/liter), penicillin (100 units/ml), streptomycin (100 μg/ml), and heat-inactivated fetal calf serum (20%). A density of 0.8 to 1.5 $\times$ 10^6 cells/ml was maintained by regular dilution of the cultures with equal volumes of fresh medium at 48-hr intervals. Under these conditions the cell mass doubled approximately every 48 hr, and cell viability was consistently greater than 95% as determined by trypan blue exclusion.

Growth of the original Raji cell stock (Raji-S) was inhibited by concentrations of BUdR above 20 μM (13). Cells resistant to 260 μM BUdR (Raji-R) were obtained by exposing the cultures to incremental concentrations of analog as reported by Hampar *et al.* (17). Development of BUdR resistance was paralleled by increased sensitivity to the hypoxanthine-aminopterin-thymidine medium described by Littlefield (25) for selection of cells deficient in TdR kinase (see "Results").

Viable cells were counted in a hemacytometer chamber using the trypan blue exclusion test. For mitotic index, drops of cells were air-dried on glass slides, fixed in neutral formalin, and stained by the Feulgen method. Approximately 500 cells were counted on each slide.

Reagents. Culture media and supplements were obtained from Grand Island Biological Co. Dimethylsulfoxide was obtained from Matheson, Coleman, and Bell, East Rutherford, N. J. Other chemical reagents were obtained from Sigma Chemical Co., St. Louis, Mo. The [^{3}H]TdR (specific activity, 20 Ci/mmole) and [^{3}H]BUdR (specific activity, 25 to 30 Ci/mmole) were obtained from New England Nuclear,

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[2] Present address: Polish Academy of Sciences, Wroclaw, Poland.

[3] Presenter. To whom requests for reprints should be addressed.

[4] The abbreviations used are: TRI, tubuloreticular inclusions; BUdR, 5-bromo-2'-deoxyuridine; TdR, thymidine; EBV, Epstein-Barr virus; IUdR, iododeoxyuridine.

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Boston, Mass. Horse and goat antisera to human globulins and other serum proteins were obtained from Behring Diagnostics, Inc., Woodbury, N. Y.

Cell Synchronization Procedures. Two-stage synchronization with a double TdR blocking technique followed procedures established by Galavazi *et al.* (10) and modified for lymphocytic cell lines by Zielke and Littlefield (41). Cells were adjusted to a density of 1.0×10^6/ml in medium containing 3 mM TdR for the 1st block. After 22 to 24 hr, cells were washed 3 times before resuspension in warm recovery medium (growth medium supplemented with 22 μM deoxycytidine). A 2nd block with either 3 mM TdR or 2.5 mM hydroxyurea was applied 10 hr later at a time when all cells were out of S phase, as determined by autoradiography and liquid scintillation counts of [^{3}H]TdR incorporation. The peak mitotic index (10 to 20%) occurred between 8 and 12 hr after release of the cells from a single 24-hr exposure to 3 mM TdR.

Experimental Treatments. After sedimenting from the final wash, synchronized cells were resuspended directly into medium supplemented with experimental reagents. Culture densities were maintained in a range of $1 \pm 0.2 \times 10^6$ cells/ml. The synchronized cells were retained in 250-ml spinner flasks with double sidearm and serum takeoff (Bellco, Vineland, N. J.), or replicate volumes (10 to 20 ml) were dispensed into 75-sq cm plastic flasks. Samples were removed aseptically from spinner flasks with disposable syringes by means of an 18-gauge needle inserted through a takeoff diaphragm at the flask base.

Measurements of Radioisotope Incorporation. Aliquots of cells were sedimented at $455 \times g$ for 10 min, resuspended in ice-cold phosphate-buffered saline (0.68 g NaCl, 0.16 g Na_2HPO_4, 0.03 g KH_2PO_4 per 100 ml, adjusted to pH 7.4), washed 3 times, and resuspended in equivalent volumes of growth medium containing [^{3}H]TdR (0.5 to 2 μCi/ml) for periods of 15 to 30 min. In some experiments, cumulative radioisotope incorporation was measured by adding 0.5 μCi of [^{3}H]TdR or [^{3}H]BUdR per ml directly into the spinner flask. Radioactive counts were measured in a Packard-Tri-Carb Model 2450 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Cells were trapped on 13-mm fiber filters (0.45 μm; Millipore Corp., Bedford, Mass.), according to the method of Robbins *et al.* (31).

Routine autoradiography was performed according to conventional dip-coating methods. Cells were pulsed for 30 min in growth medium containing 5 μCi of [^{3}H]TdR or [^{3}H]BUdR per ml, washed 3 times in phosphate-buffered saline adjusted to pH 7.4, spread on glass slides, air-dried, fixed in phosphate-buffered 0.1 M paraformaldehyde (2%), pH 7.4 for 24 hr, and rinsed in distilled water. After drying, the slides were dipped in nuclear track emulsion (NTB-2; Eastman Kodak, Rochester, N. Y.), exposed for 7 days at 5° in a dry atmosphere, developed with Kodak D-19, and stained with Giemsa. The percentage of radiolabeled nuclei was determined for 500 cells at each time point.

Electron Microscopy. Approximately 1×10^7 cells were sedimented in a fixed-angle centrifuge for 10 min at $780 \times g$ in a 16-mm round-bottom centrifuge tube. This formed a flat pellet, which was exposed to ice-cold 0.6% $KMnO_4$ in sodium acetate buffer (33) or to 3% glutaraldehyde in 0.1 M Sorensen's phosphate buffer, pH 7.4. The pellet was detached from the bottom of the tube to ensure even penetration. Fixation in $KMnO_4$ never exceeded 1 hr, and the pellets were immediately dehydrated in graded ethanols (33). Glutaraldehyde fixation was continued for 12 hr with postfixation in 1% OsO_4 (15, 33).

The $KMnO_4$ fixation was generally preferred, since it provided a high election contrast that facilitated thin-section counts of TRI (33). Ultrathin sections were obtained after epoxy embedding and stained with 5% uranyl acetate in absolute methanol followed by lead citrate (15, 33). Grids were examined at original magnifications of ×4,000 to 6,000 in a Zeiss Model EM9A electron microscope or at ×5,000 to 10,000 in a Siemens Model 1 electron microscope.

TRI Counts. At each time point, no fewer than 200 random thin sections of different cells were screened in duplicate as "unknowns." We routinely excluded any cell that was partially obscured (14) or cell fragments of less than 4-μm dimension.

A simple estimate of the minimum number of TRI in whole cells can be obtained on the premises that integral numbers of spatially defined TRI are produced at random within the cell population samples and that they will appear as integral counts in sections that are thin (circa 100 nm) in relation to the observed TRI dimensions. These premises appeared correct in practice; rarely was more than 1 TRI found per section of an individual cell. Although TRI are typically ellipsoid in contour, calculation of a minimum estimate is simplified by counting each TRI as if it were a sphere with a diameter computed from the mean maximum dimension measured on electron micrographs. This sphere should occupy a space exceeding that of any real inclusion ($p < 0.01$). The diameter (d) of the hypothetical sphere is given by $\bar{s} = \pi d/4$, where $\bar{s}$ is the average secant obtained in multiple random slices (for present purposes we substitute the calculated maximum limit of TRI dimensions).

The estimate is based upon Chart 1, which represents the hypothetical sphere of mean diameter d within a cell of

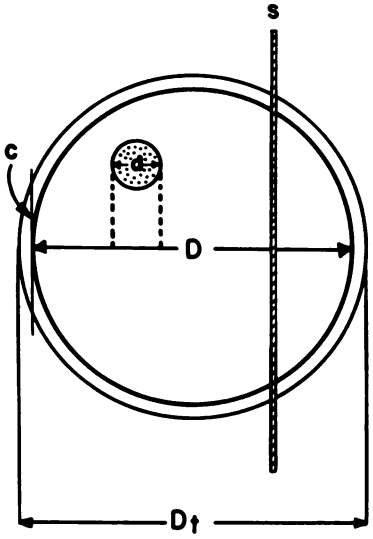

Chart 1. Geometric representation of a hypothetical TRI with diameter d in cell of effective diameter D. The total cell diameter D_t is reduced by a factor related to the length of a chord c below which cell sections are too small for inclusion in counts. The total number of parallel sections that can pass through the inclusion is equal to d/s, where s is an average section thickness.

mean diameter D_t. The effective cell diameter is corrected to D, since TRI do not localize immediately beneath the plasmalemma, and no slice smaller than a minimum chord c is counted. The average D_t of 13.5 μm was obtained by sizing BUdR-treated Raji cells in a Coulter Model T (Coulter Electronics, Hialeah, Fla.) and was close to the independently reported values of Clements *et al.* (7). Using a value of 4 μM for c, D = 13 μm is computed from the Pythagorean theorem.

When the cell is serially transected by exceedingly fine sections of average thickness s, the number of parallel sections equals D/s, and the number through each inclusion equals d/s. The probability, p, for any random section to transect an inclusion becomes $p = d/D$ and follows a binomial distribution. Use of a maximum limiting value for d gives the highest probability and thus yields the most conservative estimate of the number of TRI per whole cell. The nuclear volume need not be considered, since each transection is referred to a perpendicular plane.

High-Resolution Autoradiography. Raji cells were incubated with [^{3}H]BUdR or [^{3}H]TdR (5 μCi/ml) in a final concentration of 65 to 260 μM BUdR for induction of TRI. At intervals from 8 to 48 hr the cells were washed with drug-free growth medium containing unlabeled TdR (10^{-6} M). After 4 days the cells were sedimented at 780 × g and prepared for electron microscopic examination according to procedures described previously (15). Exposure for 7 to 14 days was sufficient to obtain heavy labeling of nuclear chromatin.

Tests for Virus Replication. In an effort to determine whether TRI might represent a stage of myxovirus replication, as postulated in some earlier reports (see Refs. 2, 16, and 29), aliquots of 0.1 ml containing approximately 5 × 10^5 TRI-positive (counts exceeding 20%) or control Raji cells were inoculated into the allantoic cavity of 7- and 10-day-old chick embryos. The embryos were incubated at 35° for 96 and 120 hr and then cooled at 4° for 4 hr. Allantoic fluid harvests failed to agglutinate guinea pig erythrocytes by the microhemagglutination technique (1) using a dextrose-gelatin-Veronal buffer as diluent.

Similar samples were inoculated in duplicate into tissue culture tubes containing the cell types: RIT-232 (a continuous rabbit liver line), Vero (a continuous African green monkey kidney line), WI-38 (diploid human embryonic fibroblasts), and primary human embryonic liver. These cells were all suppplied by courtesy of Hope Hopps, Bureau of Biologics, U. S. Food and Drug Administration, and were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 0.3% glutamine, 100 units of penicillin, and 100 μg of streptomycin per ml. Medium was changed at 24 hr after inoculation and every 3 to 4 days thereafter. Cultures were examined daily for cytopathic effects and hemadsorbtion capacity (3).

Immunofluorescent tests for intracellular EBV antigens were performed with acute-stage mononucleosis sera as previously described (23).

Tests for Intracellular Immunoglobulin. It has been suggested that TRI could be related to immunoglobulin synthesis (39). Although Raji cells consistently appear negative for intracellular IgG, tests were repeated after the BUdR treatments leading to induction of TRI. Acetone-fixed (10 min at 0°) smears were prepared for immunofluorescent examination with goat antiserum to human IgG and IgM, and crude cytoplasmic extracts (6 mg of protein per ml) from Raji cells with at least 20% counts of TRI induced by BudR were subjected to quantitative radial immunodiffusion and immunoelectrophoresis (10-μl samples) against a battery of commercially prepared goat or horse antisera to human immunoglobulins: IgG, IgM, IgD, IgA, IgE, K-chain, and L-chain.

Irradiation. Cell suspensions in 75-sq cm Falcon flasks were exposed for 2 hr to visible light from tubular fluorescent lamps at 12 cm or to a single dose of X-ray under the exact physical conditions described by Teich *et al.* (38) for murine leukovirus induction, conditions similar to those used by Fogel (9) for polyoma virus induction.

RESULTS

TRI Induction in Synchronized Cells. Synchronization of Raji cells with successive 3 mM TdR blocks produced results similar to those previously described in lymphoid cell lines (18, 41). A typical experiment is illustrated in Chart 2. The rate of [^{3}H]TdR incorporation measured was maximal within 1 hr after release from the 2nd TdR block and fell off to a minimum level coincident with the rise in mitotic index at 8 to 10 hr. Similarly, more than 90% of cells examined by autoradiography were pulse-labeled by [^{3}H]TdR within 2 hr, and this ratio fell to 20% before mitosis. Synchronization of untreated cells persisted for at least 2 cell cycles. The rate of

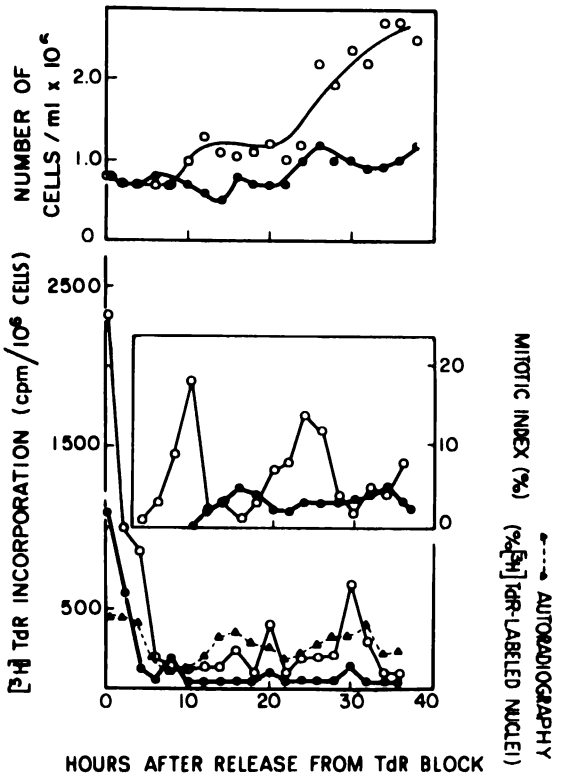

Chart 2. Typical cell-cycle kinetics in Raji cells after a 2-stage TdR synchronization. Upon release from the 2nd block, cells were treated with 260 μM BUdR (●) or TdR (○). Samples removed at regular intervals were washed and pulse-labeled with [^{3}H]TdR (1.92 Ci/mole) for 30 min and prepared for liquid scintillation spectrometry or autoradiography (▲, shown for TdR). Mitotic index was determined from unlabeled samples. Counts in a control without TdR (not shown) closely paralleled the plots shown for TdR.

[^{3}H]TdR incorporation into cells exposed to 260 μM BUdR was similar to that for controls during the 1st S phase, although there was a delayed rise in the mitotic index. The 2nd cycle of [^{3}H]TdR incorporation into BUdR-treated cells was not sharply defined, but there was a prolonged increase in mitoses.

Cell sections were examined for evidence of TRI induction at intervals after termination of the 2nd TdR block and the beginning of exposure to 260 μM BUdR for a single S phase (zero time). Minimal numbers of inclusions appeared as early as 36 hr, but the maximal counts (20 to 35% of cell cross-sections) were observed between 48 and 96 hr (Chart 3, *top*). This is in comparison with a 48- to 72-hr time lag noted earlier in randomly growing cultures (13, 36). The density of viable cells reached a plateau for an interval during TRI formation (Chart 3, *bottom*). Cell growth typically resumed within 96 hr after the single-cycle BUdR exposure. Cells with TRI appeared cytologically intact and were occasionally seen in mitosis as noted also by Splinter *et al.* (36).

When cycloheximide, a reversible inhibitor of polypeptide translation, was introduced between 36 and 48 hr, the induction of TRI was suppressed, and there was an additional 48-hr lag before recovery from this effect (Chart 3, *top*). In these experiments, cell growth declined irreversibly (Chart 3, *bottom*).

Further experiments indicated that continuous cell division was not prerequisite to the TRI induction. After a 2-stage synchronization with TdR, cells were exposed to 260 μM BUdR for a single S-period (8 hr). The cells were washed and immediately reblocked with 3 mM TdR for 72 hr, at which time electron microscopy disclosed 13% TRI. After 36 hr in BUdR, synchronized cultures were exposed to colchicine (2 μg/ml) for 24 hr, and up to 10% TRI were found in sections of cells arrested at metaphase.

Estimate of TRI in Whole Cells. The maximum dimensions of randomly selected TRI were measured on calibrated electron photomicrographs of ultrathin sections from several different experiments after glutaraldehyde (33 sections) or $KMnO_4$ fixation (28 sections) (Fig. 1). The maximum dimensions (mean ± S.D.) were 1.80 ± 0.61 μm and 1.78 ± 0.69 μm, respectively. A theoretical maximum limit was calculated by using each mean + 3 S.E., yielding values of 2.7 and 2.8 μm. The minimum TRI frequency was then calculated, as described in "Materials and Methods," using a value of 2.8 μm as the maximum TRI dimension (d) and an effective cell diameter (D) of 13 μm.

Using a binomial probability table we estimated that TRI counts above 34 per 200 individual lymphoma cell sections (17%) reflected no less than 1 TRI per 2 whole cells ($p < 0.05$). In untreated Raji cell cultures TRI were never observed, and the controls were counted in sufficient numbers (>2000 sections) to indicate that positive experimental findings were significant.

Relationship of TRI Induction to S Phase. Experiments consistently demonstrated that exposure to BUdR during a single S phase was sufficient to induce a high range of TRI counts within 96 hr. Indeed, there was little difference in the level of TRI induction observed in cultures exposed for a single S phase as compared to cultures exposed continuously to BUdR (Table 1). Periods of BUdR exposure up to 8 hr (Chart 4) were within a single cycle of DNA synthesis, as shown both in Chart 2 and in comparable studies by Zielke and Littlefield (41). Exposure to 260 μM BUdR during limited periods after the peak of S phase produced a minimal induction of TRI (Chart 4), which could be explained by cell cycle variability and loss of synchrony. An overall correlation between the level of TRI induction and the total incorporation of BUdR was suggested by the trend of these data, as well as results with increasing concentrations of BUdR (Table 1). Counts of TRI (Table 2) obtained during experiments illustrated in Chart 2 also showed a correspondence to BUdR exposure during periods of the cell cycle when the incorporation into DNA should have been maximal. Direct quantitation of BUdR incorporation during each time interval remains to be performed; however, previous work with Raji cells by Henderson and Strauss (19) showed efficient DNA base substitution under very similar experimental conditions.

In further experiments, the inhibitor hydroxyurea, which does not compete with BUdR for molecular position in the

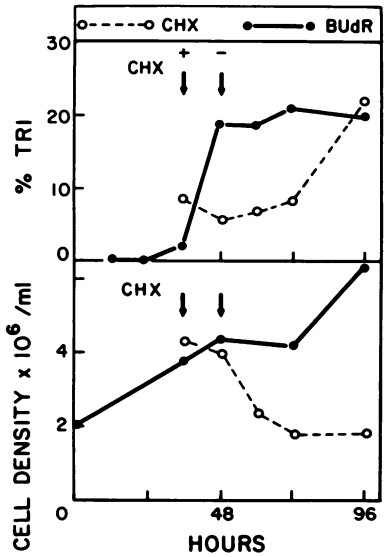

Chart 3. *Top*, time course of TRI induction in Raji cells after exposure to BUdR. The cells were synchronized with a 2-stage TdR block, exposed to 260 μM BUdR for 8 hr, and washed. One set of spinner flasks was treated with cycloheximide (*CHX*) (100 mg/ml) at 36 hr, and the drug was washed out at 48 hr (*arrows*). TRI counts are for 200 random cell sections at each *time point*. *Bottom*, changes in cell density after BUdR and cycloheximide treatment.

Table 1

Induction of TRI by serial concentrations of BUdR or IUdR

In 1 set of parallel experiments, unsynchronized Raji cells were continuously exposed to increasing concentrations of halogenated pyrimidine for 96 hr. In a 2nd set, the cells were synchronized twice with 3 mM TdR exposed for 8 hr, washed, and examined at 96 hr.

Concentration of analog (μM)	TRI counts/200 cell sections (%)			
	Unsynchronized cells		Synchronized cells	
	BUdR	IUdR	BUdR	IUdR
None	0	0	0	0
16	0	7	2	0
33	3	6	13	3
65	14	12	17	5
130	22	20	21	13

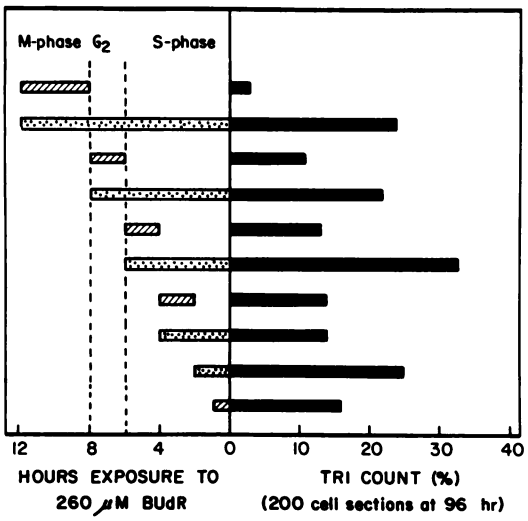

Chart 4. Induction of TRI in Raji cells at 96 hr, related to durations of exposure to 260 μM BUdR after 2-stage TdR synchronization (*cf.* Fig. 1). *Patterned bars* at *left*, exposures in 2 separate experiments; *dashed lines* on *left*, estimated positions of the S-, G_2-, and M-phase boundaries.

Table 2

Correlation of TRI counts with exposure of synchronized Raji cells to 260 μM BUdR during sequential 4-hr intervals of 2 cell cycles

Same conditions as in Chart 2. Cells were treated with BUdR for indicated intervals, washed, and placed in fresh medium with 10^{-6} TdR.

Interval (hr)	Phase of cycle	TRI counts (%)[a]
0–4	Early S	14
4–8	Late S, G_2	2
8–12	G_2, M	0
12–16	Early S	16
16–20	Late S, G_2	6

[a] Samples 96 hr after termination of 2nd TdR block, percentage of 200 cell sections.

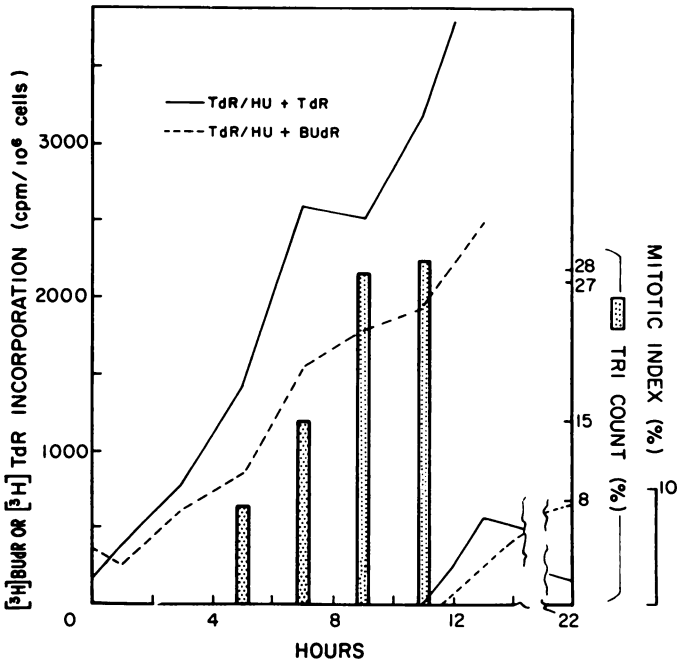

Chart 5. Cumulative incorporation of [³H]BUdR or [³H]TdR into Raji cells entering S phase after a 2-stage block, with TdR and hydroxyurea (*TdR/HU*). Cells at a density of 1.0 × 10^6/ml were maintained in separate spinner flasks with 260 μM TdR or BUdR, as shown, and [³H]TdR or [³H]BUdR (1.92 Ci/mole). Samples were removed at regular intervals and prepared for liquid scintillation spectrometry, electron microscopy, and mitotic indices. The TRI counts were performed at 96 hr (*bars*).

DNA strand (5, 28) was used as the 2nd-stage blocking agent. Under these conditions the S phase terminated at 7 to 9 hr, and the mitotic index peaked between 11 and 12 hr after release from the 2.5 mM hydroxyurea block. A maximum number of TRI were observed by exposure of the synchronized cells to 260 μM BUdR for approximately 10 hr (Chart 5), and a direct comparison of TRI induction to the amount of BUdR incorporation was obtained by measuring cumulative counts of [³H]BUdR or [³H]TdR at regular intervals (Chart 5). This approach complemented pulse-label data of the type shown in Chart 2. Results were again consistent with the interpretation that TRI induction depends upon substitution of bromouracil into the newly formed DNA.

High-Resolution Autoradiography. Direct evidence for incorporation of BUdR into the DNA of Raji cells producing TRI was obtained by exposing cells to [³H]BUdR for up to 48 hr. In high-resolution autoradiographs, silver grains were preferentially localized over the interphase nuclear chromatin or the chromosomes of dividing cells. Silver grains never concentrated above the TRI in comparable fields (Fig. 2). Indirectly, this indicated an absence or paucity of nascent DNA in the TRI, a finding compatible with earlier cytochemical findings of TRI resistance to nucleases (33).

BUdR-resistant Cells. Association of the BUdR effect with DNA synthesis was further supported by tests of a Raji cell mutant culture (Raji-R) that had been selected for ability to grow in progressively increasing concentrations of BUdR over a period of 5 months (see "Materials and Methods"). This subline failed to survive in selective hypoxanthine-aminopterin-thymidine medium, and in contrast to Raji stock cultures their growth was not inhibited by excess BUdR or TdR (260 μM). No TRI were found in numerous samples (>10^3 sections) of the Raji-R cells treated continuously with 33 to 260 μM BUdR.

Pyrimidine Supplementation. Halogenated pyrimidines can cause a significant deoxycytidine deficiency by toxic inhibition of ribonucleotide reductase. Meuth and Green (27) argued that such inhibition could account for certain effects attributed to bromouracil substitution. To test this possibility, synchronized cells were treated with varying molar proportions of BUdR and deoxycytidine. Even in equimolar ratios, BUdR induced an intermediate range of TRI counts. In contrast, increasing amounts of TdR, which competes metabolically with BUdR and thus decreases its substitution into DNA, produced a progressive inhibition of TRI induction (Chart 6).

Concentration Dependence. In cultures continuously exposed to BUdR, a threshold concentration of approximately 16 μM was required for the detection of TRI, but a high range of counts was not achieved below 65 μM. Continuous exposure to 8 μM BUdR for as long as 8 weeks did not produce an observable level of inclusions. In synchronized cultures treated for a single S phase, there also appeared to be generally higher TRI counts correlated with increasing BUdR or IUdR concentrations (Tables 1 and 3).

Comparison of IUdR and BUdR. Induction of TRI after continuous treatment of Raji cells with with several concen-

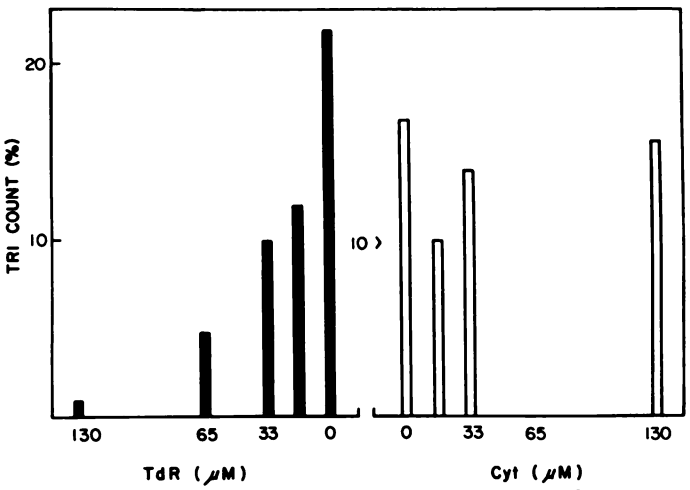

Chart 6. Effects of pyrimidines on TRI induction. After a 2-stage synchronization with TdR, Raji cells were treated with 130 μM BUdR plus the indicated concentrations of either TdR or deoxycytidine (*Cyt*) for 0 to 8 hr, then washed and maintained in medium with 16 μM TdR or deoxycytidine. TRI counts (*bars*) were performed at 96 hr after start of the BUdR treatment.

Table 3

TRI induction in the presence and absence of visible light

Raji cultures synchronized twice with 3 mM TdR were exposed to indicated concentrations of BUdR for 8 hr and washed under dark conditions. Half of the flasks and an untreated control flask were exposed to 40-watt fluorescent light at 10 cm for 2 hr beginning 24 hr after zero time; they were then handled under ambient lighting conditions. The remaining flasks, spray-coated with black paint and covered with aluminum foil, were exposed to negligible light during a single feeding at 48 hr. Samples were prepared for electron microscopy at 96 hr.

Concentration of BUdR (μM)	TRI counts/200 cell sections (%)	
	Light exposed	Unexposed
None	0	0
16	3	2
33	15	15
65	20	19
130	23	24

trations of IUdR or after the treatment of synchronized cells with IUdR for a single S phase was quantitatively similar to induction by BUdR (Table 1).

Related Studies. Visible light had no significant effect on the induction of TRI through the entire range of BUdR concentrations (Table 3). This contrasted to virogenic effects of BUdR, which can be enhanced by photoactivation (9, 38). In companion experiments (not shown) X-radiation in amounts up to 1000 R, alone or after BUdR exposure, also produced no detectable stimulation of TRI induction but was progressively cytotoxic.

Synchronized Raji cells were exposed to 1 or 2% dimethylsulfoxide with and without several concentrations of BUdR, but again no effect on TRI induction or cell growth was discernible.

Tests for intracellular immunoglobulin in BUdR-treated Raji cells were negative.

Virological Studies. Monolayer cultures inoculated with TRI-positive Raji cells and examined daily showed no cytopathic effect. Hemadsorption tests for possible myxovirus antigens were negative at 4, 8, and 10 days at both 4° and 22° with cockerel, guinea pig, human O-negative, and sheep erythrocytes. Allantoic fluid harvested from embryonated eggs inoculated with TRI-positive Raji cells failed to agglutinate guinea pig erythrocytes. Indirect immunofluorescent assays for intracellular measles virus (4) using hyperimmune monkey antisera also were negative.

Antisera from patients with infectious mononucleosis confirmed an expected activation of intracellular EBV antigens in BUdR-treated Raji cells (11, 18, 21). These antigens appeared more rapidly than did TRI, as has been observed previously (18, 21). Only a small fraction of cells is involved (11, 21), and the distribution did not suggest specific localization to TRI. By electron microscopy, EBV nucleocapsids were never observed in Raji cells during the TRI induction.

DISCUSSION

Accumulation of tubular complexes within the endoplasmic reticulum of lymphocytes in patients with immunological or neoplastic diseases, including malignant lymphoma, has been widely reported (6, 14, 16, 20, 30). Ultrastructurally and cytochemically, these lymphocyte inclusions appear to be indistinguishable from TRI induced experimentally in the human lymphoma cell cultures (33). Thus the lymphoma cell system may provide a useful pathobiological parallel for the thus far unexplained clinical abnormality. In other mammalian cells, phenotypic changes produced by halogenated pyrimidine analogs typically have been related to reversible DNA base substitution and a subsequent nonmutagenic influence at the level of RNA transcription (32). A virogenic potential of BUdR has been attributed to derepression of viral genetic information which is otherwise restricted by host controls (26, 38). A primary effect on regulatory genes has been proposed, since these are represented in the repetitive DNA sequences that appear to be preferentially substituted with low concentrations of [^{3}H]BUdR (34). Lin and Riggs (24) were able to demonstrate an exponential decrease in the rate of dissociation of *lac* repressor from BUdR-substituted *lac* operator gene as compared to unsubstituted DNA.

Data from experiments of Henderson and Strauss (19) indicate that up to 90% of the total thymine bases in Raji-cell DNA can be replaced during a continuous BUdR treatment of 96 hr. Such a high degree of substitution evidently is not necessary for induction of TRI. Incorporation of halogenated pyrimidine during times equivalent to a single S-period, or even fractions of this period, induced TRI to approximately the same extent as previously observed under conditions of continuous analog exposure. The model of semiconservative DNA replication predicts that halogenated pyrimidine bases incorporated during a single S-period should be located in only 1 of the complementary DNA strands in each pair of daughter cell chromatids. According to the report cited (19), 30 μM BUdR, which proved sufficient to induce minimal TRI after 1 S-period, replaces no more than 60% of thymine residues in replicated DNA strands.

In our experiments, single-strand halogenated base substitution evidently exerted a "unifilar dominant" effect similar to that observed on induction of hepatoma tyrosine aminotransferase (37) and *lac* repressor binding (24). The dominance may be attributed to replacement inhibition of

transcriptional processes dependent upon the enzymatic recognition of paired normal DNA strands (32). Formation of TRI despite inhibition of DNA replication by excess TdR or during blockage of karyokinesis by colchicine after the BUdR treatment indicated that transcription of an altered DNA need not precede the induction. A similar conclusion was drawn by Splinter *et al.* (36), since cytosine arabinoside failed to prevent induction of TRI in EB-3 lymphoma cells.

The significance of our *in vitro* findings in relation to the clinical observations of TRI in blood or tissue lymphocytes of patients with neoplastic and immunological diseases remains to be elucidated. Cytochemical studies indicate that both the clinically observed and the experimentally induced TRI may contain a common mucoproteinaceous component that appears to be membrane associated (33). More definitive biological identification awaits success of ongoing efforts to separate this material. For the present, our results seem consistent with either of 2 lines of reasoning.

1. The formation of TRI could result from an altered pattern of cellular gene expression analogous to that observed in other mammalian systems after bromouracil incorporation into DNA. Phenotypic effects may be manifested during cytodifferentiation (32) and in mature cells (40), neoplastic cells (35, 37), or virus-transformed cells (12). Considering an effect of BUdR on transferase enzymes noted by Stellwagen and Tomkins (37) and by Walther *et al.* (40), it seems conceivable that TRI might represent an incompletely glycosylated mucoprotein which condenses abnormally in the cytosecretory apparatus.

2. TRI could represent a product of latent virus genes or reflect a virus-host cell interaction. Although it is now widely accepted that TRI differ ultrastructurally from any known class of animal virion (8, 16, 29, 33, 39), an indirect relationship to virus replication has not been dismissed (16, 29, 39). In cultured lymphoid cells, for example, the possibility that TRI induction relates to the concomitant expression of EBV antigens after BUdR treatment (11, 18, 21) requires additional study. Thus far, induction of maximal numbers of TRI has succeeded primarily in EBV-positive B-cell lines (16); nevertheless, minimal numbers were identified in at least 1 lymphoma cell line, which is reported negative for EBV genome by the complementary hybridization technique (22). In an earlier study, no correlation was found between the presence of TRI in blood lymphocytes and the serum EBV antibody titers (23). An association of TRI and measles virus infection also has been considered (2, 20, 29), but present and earlier tests (29) for myxovirus antigens have been uniformly negative in cells with TRI. Infection of Raji cells with measles virus (4) also failed to induce TRI (16).

Within binomial probability limits imposed by thin-section sampling, the *in vitro* lymphoma cell system for induction of TRI by halogenated pyrimidines offers repeatable conditions for continued experimental investigations. This should facilitate efforts to test selective metabolic inhibitors or to detect specific viral genes (22) or replicases (26). Preliminary work indicates that TRI remain partially intact after bulk disruption of induced Raji cells in hypotonic buffer. Density gradient fractionation may eventually permit more definitive biochemical or immunological characterization.

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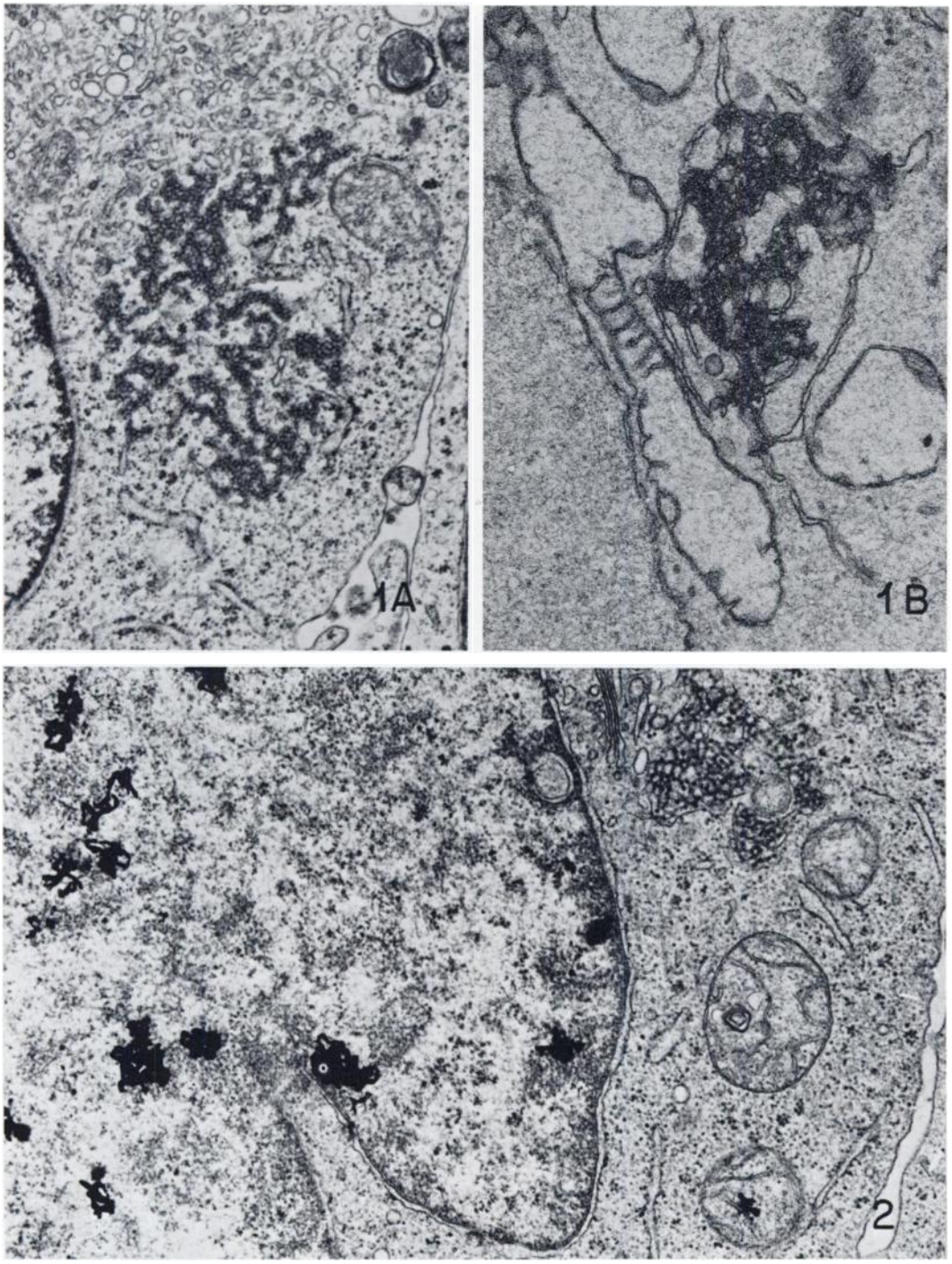

Fig. 1. Thin sections of TRI in BUdR-treated Raji cells fixed with glutaraldehyde (*A*) and $KMnO_4$ (*B*). × 17,200.

Fig. 2. Autoradiograph of a Raji cell exposed to [^{3}H]BUdR (77 Ci/mole) during continuous treatment with 65 μM BUdR for 4 days. TRI counts in this experiment were 20%. The thin sections were exposed to emulsion for 2 weeks. The developed grains were localized in the nucleus and rarely in a mitochondrion (*lower right*); they were never found above TRI (*upper right*). × 23,000.