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Research Article

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Selective Release of Excreted DNA Sequences from Phytohemagglutinin-Stimulated Human Peripheral Blood Lymphocytes

EFFECTS OF TRYPSIN AND DIVALENT CATIONS

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ABSTRACT We studied the synthesis of excreted DNA sequences and their release from phytohemagglutinin-stimulated human peripheral blood lymphocytes under conditions permitting optimal cell growth. Cells were labeled by constant exposure to low specific activity [^{3}H]thymidine. Excreted DNA sequences were synthesized during the period of logarithmic cell growth and moved slowly from the high molecular weight chromosomal DNA fraction into the low molecular weight cell DNA fraction (Hirt supernate) from which they could be specifically released by treating the cells briefly with small amounts of various proteases; 1 μg/ml trypsin for 5 min was optimal. On day 5 of culture, 13.3±6.9% of the total cellular acid-precipitable [^{3}H]thymidine was released by this treatment. Trypsin-induced release was partially and reversibly inhibited by incubating the cells for 16 h with 5 mM dibutyryl-cyclic AMP. Cells incubated in the absence of divalent cations spontaneously released this Hirt supernatant DNA; after maximal release had occurred under these circumstances, additional trypsin treatment caused no further release of DNA. Trypsin-induced DNA release could be completely and reversibly inhibited by incubating the cells in the presence of 10 mM calcium.

Trypsin-released DNA was isolated and analyzed by reassociation kinetics. A major component, representing 54% of the DNA, reassociated with a $C_0t½$ of 68 mol·s/liter (the value at which DNA association is 50% complete). The reassociation of this DNA was studied in the presence of an excess of DNA isolated from stimulated lymphocytes on day 3 in culture, and in the presence of an excess of resting lymphocyte DNA. The high molecular weight fraction of day-3 cell DNA contained three times more copies of the trypsin-released DNA major component as compared to resting lymphocyte DNA. Hirt supernatant DNA isolated from day-5 stimulated lymphocytes reassociated in an intermediate component representing 34% of the DNA with a $C_0t½$ of 26 mol·s/liter; after cells were treated with trypsin, this component could no longer be identified in the Hirt supernatant fraction, presumably because it had been released into the incubation medium.

These data describe a quantitatively reproducible system with which synthesis and release of excreted DNA sequences can be studied.

INTRODUCTION

Human peripheral blood lymphocytes, stimulated in vitro with phytohemagglutinin (PHA),[1] release a portion of their newly synthesized DNA into the culture medium (1–3). Our previous studies demonstrated that a large part of this DNA is composed of single-copy sequence elements that reassociate in a major component representing a complexity of about 10% of the genome, and that lymphocytes derived from different donors released largely the same sequences (2). We reasoned that these DNA sequences might result from selective replication of a limited portion of the lymphocyte genome. Alternatively, the limited complexity seen in DNA released into the culture medium theoretically could represent restricted degradation of cellular DNA by nucleases so that only the same limited number of sequences would remain as the end product of cell death. To test these hypotheses, we quantitated

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[1] *Abbreviations used in this paper*: C_0t, concentration of DNA nucleotides multiplied by time of incubation and expressed as moles·seconds/liter (mol·s/liter); $C_0t½$, that value at which DNA reassociation is 50% complete; DB-cAMP, dibutyryl-3′,5′-cyclic adenosine monophosphate; [^{3}H]dT, [^{3}H]thymidine; HBSS, Hanks' balanced salt solution; PHA, phytohemagglutinin; RMS, root mean square.

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the number of copies of sequences comprising the major reassociation component in culture medium DNA per haploid genome at various times during the culture period, because selective replication would require that extra copies be present (3). After 3 and 4 days of culture, the high molecular weight fraction of cell DNA apparently contained three- to fourfold more copies of these sequences when compared with resting lymphocyte and to placenta DNA. By day 6 of culture, this number declined to a one- to twofold difference (3). As the number of copies present in high molecular weight form declined, the same sequences appeared first in the Hirt supernatant (low molecular weight) fraction of cell DNA and then in the culture medium (3). These data suggest that PHA-stimulated lymphocytes selectively replicate extra copies of a limited portion of their genome; initially, the extra copies are associated with high molecular weight DNA but later move into the Hirt supernatant fraction. Presumably, at that stage they are ready for release into the culture medium. We designated these sequences as "excreted" DNA to distinguish them from DNA that might be nonspecifically released by cell lysis.

The work presented here was designed to test our hypothesis that low molecular weight sequences present in the Hirt supernate (3) were precursors to those later appearing in the culture medium. The present experiments are organized as follows: first, conditions are defined under which cell growth and viability are optimal, so that DNA potentially released by dead cells could be minimized. Next, manipulations that cause Hirt supernatant DNA to be released into the incubation medium are described, namely, protease treatment and exposure to divalent cation-free buffers. Subsequent experiments assess the specificity of DNA release by comparing this event to release of other cell components from PHA-stimulated lymphocytes, and by measuring release of DNA under similar conditions from two different diploid human culture cell lines. The final experiments describe the kinetic complexity of trypsin-released DNA and of Hirt supernatant DNA.

The work demonstrates that Hirt supernatant DNA contains the immediate precursor to excreted DNA appearing in the culture medium. We have defined conditions that induce selective release of these precursor molecules and that inhibit their release. Under optimal culture conditions we show that this phenomenon is quantitatively reproducible.

METHODS

Materials. All chemicals were reagent grade. Hydroxyapatite (Bio-Gel HTP) was from BioRad Laboratories, Richmond, Calif. Minimum Essential Medium-Alpha (MEM-α) was obtained from Flow Laboratories, Rockville, Md. Penicillin and streptomycin were purchased from Grand Island Biological Co., Grand Island, N. Y. All radioisotopes were purchased from New England Nuclear, Boston, Mass. Leukoagglutinating and erythroagglutinating phytohemagglutinin of *Phaseolus vulgaris* were purified from PHA-P (Difco Laboratories, Detroit, Mich.) by the method of Weber et al. (4). Conconavalin A was purchased from Calbiochem, San Diego, Calif., and a lectin from the mushroom *Agaricus bisporus*, prepared as described (5), was the gift of Dr. C. Presant (Washington University, St. Louis, Mo.). Cytochalasin B and the calcium ionophore A23187 were gifts of Dr. C. Parker (Washington University, St. Louis, Mo.). Valinomycin, colchicine, dibutyryl-3′,5′-cyclic adenosine monophosphate (DB-cAMP), pronase (Protease, type VI), trypsin (type III, twice crystallized from bovine pancreas), RNase A, cetrimide (cetyltrimethyl ammonium bromide), soybean trypsin inhibitor, thymidine, lactic dehydrogenase, NADH, sodium pyruvate sodium lauroyl sarcosine, and salmon sperm DNA were purchased from Sigma Chemical Co., St. Louis, Mo. Powdered polyethylene glycol (Carbowax 6000) was obtained from Union Carbide, New York. Vincristine was purchased from Eli Lilly Co., Indianapolis, Ind. Hanks' balanced salt solution (HBSS; 6) was prepared according to the formula 406 in the Grand Island Biological catalogue (1976–77). DNase I (RNase-free) was purchased from Worthington Biochemical Corp., Freehold, N. J. Purified *Escherichia coli* DNA polymerase I (fraction VII) was the generous gift of Dr. Lawrence Loeb (Institute for Cancer Research, Foxchase, Philadelphia, Pa.). Human plasminogen and streptokinase were the gifts of Dr. N. Alkjaersig, bovine and human thrombin were the gifts of Dr. Craig Jackson, and human Factor X_a was the gift of Mr. J. Miletich and Dr. P. W. Majerus (all of Washington University School of Medicine, St. Louis, Mo.). Diploid human fibroblasts were the gift of Dr. W. Sly (also of Washington University). H-SB2 lymphoblasts were the gift of Dr. C. Parker.

Lymphocyte cultures. Human peripheral blood lymphocytes were isolated as described (7) except that the leukocyte-rich supernate from the Dextran sedimentation step was not diluted further. Cells were cultured by the method of Stewart and Ingram (8) at an initial concentration of 300,000–500,000 cells/ml in MEM-α supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and 15% autologous serum. Cells were cultured in 2-ml vol in 15-ml screw cap plastic tubes or in 50-ml vol in 250-ml (75 cm^2) plastic tissue culture flasks purchased from Falcon Plastics, Div. of BioQuest, Oxnard, Calif. Cultures were stimulated on day zero by adding leukoagglutinating phytohemagglutinin at a concentration of 2.5 μg/ml, and were maintained for up to 8 days in a 5% CO_2 atmosphere at 37°C.

Method of counting cells. Cell counts were performed by the pronase-cetrimide technique of Stewart et al. (8, 9). Duplicate 2-ml cultures were dispersed and incubated for 15 min at 37°C with an equal volume of 5 mg/ml pronase to digest nonviable cells and debris. The cells were then suspended in 10 ml of cetrimide, and the nuclei counted at the red cell setting on a model F Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.).

DNA labeling. Lymphocytes were continuously labeled by adding a sterile solution of [*methyl*-^{3}H]thymidine (2 Ci/mmol diluted to 50 mCi/mmol with cold thymidine) at a concentration of 0.25 μCi/ml on day zero.

Measuring DNA

Media and cells. In initial experiments, we measured acid-precipitable radioactivity in culture media and cells as described previously (1) except that NCS reagent (Amersham/Searle Corp., Arlington Heights, Ill.) digests of precipitable

material were counted in a 2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]benzene (PPO-POPOP)-toluene scintillation mixture. In later experiments, 500-μl medium samples were precipitated with 5 ml 5% trichloroacetic acid in the presence of 50 μg salmon sperm DNA carrier and collected on polycarbonate membrane filters having a pore size of 0.2 μm (Uni-Pore, BioRad Laboratories, Richmond, Calif.) which were dissolved and counted in a PPO-POPOP-toluene scintillation mixture containing NCS. Both methods gave equal counting efficiency.

Hirt supernate. Cellular DNA was fractionated by the method of Hirt (10). This procedure separates cellular DNA into a pellet containing high molecular weight chromosomal DNA and a supernate containing low molecular weight DNA. Cells in individual culture tubes at 4°C were washed once with ice-cold 0.9% NaCl and transferred to 1.5-ml polypropylene centrifuge tubes (Brinkmann Instruments, Inc., Westbury, N. Y.) where they were resuspended in 0.1 ml of 0.9% sodium chloride; to this suspension was added 1 ml 0.01 M Tris-HCl pH 7.5—0.01 M EDTA—0.6% sodium dodecyl sulfate. The suspension was mixed gently by inverting the tube, allowed to sit at room temperature for 15 min, then mixed with 0.3 ml 5 M sodium chloride. The suspension was stored at 4°C for 16 h, and then centrifuged 12,000 *g* at 4°C for 20 min. The supernate was removed and precipitated with 50 μg salmon sperm DNA in 10 ml 5% trichloroacetic acid. The acid precipitates were handled as described above.

Nuclear and cytoplasmic fractionation. Nuclear and cytoplasmic fractions were separated by a modification of the method of Berger and Cooper (11). Cells were washed with HBSS and resuspended in reticulocyte standard buffer (0.01 M Tris·HCl pH 7.4–0.01 M sodium chloride-0.0015 M magnesium acetate). The cell suspension was underlayered with an equal volume of this buffer containing 9% sucrose and 1% NP40 (Shell Oil Co., Houston, Tex.) and centrifuged at 1,000 *g* for 20 min. Cells were rapidly lysed as they moved into the detergent-containing layer, and the nuclei formed a tight pellet. Both the nuclear and cytoplasmic fractions were acid precipitated, and radioactivity was determined as described above.

Preparation of proteases

Pronase was dissolved in 0.9% NaCl at a concentration of 5 mg/ml, and trypsin was dissolved in 0.01 M $CaCl_2$-0.04 M Tris-HCl pH 7.5 at a concentration of 1 mg/ml. Trypsin activity was assayed by measuring the rate of hydrolysis of *p*-toluenesulfonyl L-arginine methyl ester at 30°C by following the increase in absorbance at 247 nM.

Cell harvesting and incubations

Unless otherwise stated, 2-ml cell suspensions on day 5 of culture, containing 0.5–0.6 × 10^6 cells/ml, were used in the experiments. Cells were gently pelleted by centrifugation at 300 *g* for 10 min, washed with HBSS, resuspended in 1 ml HBSS, and then incubated at 37°C as described for each experiment. Various proteases were added to the incubation mixtures to elicit DNA release. When pronase was used, the incubation mixtures were harvested by placing them in ice, then centrifuging at 500 *g* for 10 min at 4°C to separate cells from medium. When trypsin was used, the incubation mixtures were harvested by one of two methods. In the first method, the tubes were placed in ice, fivefold excess (by weight) soybean trypsin inhibitor was added, and the cells were centrifuged at 500 *g* for 10 min at 4°C. In the second method, 1-ml cell suspensions were layered over 0.4 ml of an oil mixture in 1.5-ml polypropylene tubes before incubation. The oil mixture was composed of one part Apiezon oil (James G. Biddle Co., Plymouth Meeting, Pa.) and nine parts *N*-butylphthalate (Fischer Scientific Co., Pittsburgh, Pa.) with a final density of 1.029. To terminate incubations, cells were pelleted through the oil layer by centrifugation at 12,000 *g* for 15 s in an Eppendorf 5412 centrifuge (Curtin-Matheson Scientific, Houston, Tex.). Both methods gave similar recovery of acid-precipitable radioactivity but the latter method, which insured that protease action was stopped at the desired time interval, prevented use of the cells for Hirt supernatant preparations.

Lactic dehydrogenase measurement

Lactic dehydrogenase was assayed on samples of incubation medium and cells before and after trypsin treatment using the method of Reeves and Fimognari (12). Trypsin was inactivated by the addition of a fivefold excess of soybean trypsin inhibitor to the medium before the assay. Cells were resuspended in 1 ml 1% bovine serum albumin-0.15 M potassium chloride-0.05 M potassium phosphate, pH 7.4, and homogenized in a Plexiglas micro-tissue grinder (Belco, Vineland, N. J.). Lactic dehydrogenase activity was then assayed by measuring the rate of decrease in absorbance of NADH at 340 nM in 0.1 M potassium phosphate buffer containing 0.011 M sodium pyruvate.

^{51}Cr labeling

PHA-stimulated lymphocytes on day 5 of culture were labeled with ^{51}Cr (13) by adding 60 μCi of isotope in 60 μl 0.9% NaCl to each 2-ml culture tube. The tubes were mixed gently, then incubated 4 h at 37°C.

Purification and analysis of DNA

To purify trypsin-released DNA from the incubation medium, the medium was made 0.2% with respect to sodium lauroyl sacrosine, and 0.01 M with respect to both Tris HCl, pH 7.4, and EDTA, and concentrated at 4°C by dialysis against powdered polyethylene glycol. 8-ml aliquots of the concentrated solution were mixed with 8 g cesium chloride and 1.5 mg ethidium bromide and centrifuged at 32,000 rpm in the Beckman 50 Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 4°C for 40 h. The resulting gradients were handled in dim light; the visible red DNA bands were removed with a pipette. The DNA-containing solution was extracted twice with *n*-butanol to remove ethidium bromide, dialyzed against 0.01 M Tris-HCl pH 7.4 0.01 M EDTA, and then treated first with 50 μg/ml RNase A (previously heated 10 min at 100°C) for 2 h, 37°C, and then with pronase, 0.5 mg/ml for 2 h. The solution was made 0.25% with respect to sodium lauryl sulfate and 0.2 M with respect to sodium chloride and extracted twice with a mixture of phenol-chloroform-isoamyl alcohol (50:50:1). The aqueous phase was dialyzed against 0.02 M sodium acetate, pH 7.0, then made 0.2 M with respect to sodium acetate, pH 7.0, and DNA was precipitated at −20°C after the addition of 2 vol of absolute ethanol. Cell and Hirt supernatant DNAs were purified, and DNA purity was monitored as previously described (2, 3). All DNAs were sheared by sonication and analyzed by reassociation kinetics as described (2, 3).

RESULTS

Lymphocyte culture methods. We used the culture system of Stewart et al., (8, 9) to insure optimal cell growth. In agreement with their findings, after a fall in cell number to a nadir on day 2 or 3 of culture, PHA-stimulated human peripheral blood lymphocytes grew in log to a peak in days 5 to 6 of culture (Fig. 1). Because PHA agglutinates lymphocytes, the pronase-cetrimide method (8, 9) was used to disperse the cells for counting and for assessment of viability. Pronase treatment completely disaggregated the lymphocytes; after this treatment 97% excluded trypan blue. Cetrimide lysed the cells to permit counting of their nuclei. When cells were lysed with cetrimide without prior pronase treatment, the counts presumably represented both viable and dead cells. During the period of rapid growth and, in particular, on day 5 of culture when most of our experiments were performed, there was no difference in cell numbers obtained by counting without pronase treatment compared to counts with pronase treatment before the addition of cetrimide (Fig. 1).

We found that the serum plus 1% dextran remaining above the lymphocyte layer during Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals, Piscataway, N. J.; Hypaque, Winthrop Laboratories, New York) purification could substitute equally well for donor serum in preparing culture medium. Lymphocyte growth was similar in standard 2-ml aliquots contained in 15-ml plastic screw cap tubes and in 50-ml aliquots contained in 250-ml plastic tissue culture flasks.

Continuous exposure during growth to 0.25 μCi/ml, 50 mCi/mmol, [^{3}H]thymidine ([^{3}H]dT) did not significantly alter cell growth (data not presented).

Protease-induced release of Hirt supernatant DNA. We found that the pronase treatment used to prepare PHA-stimulated lymphocytes for cell counting resulted in loss of DNA from the Hirt supernatant fraction. As detailed in Table I, cells suspended in their original culture medium and treated with increasing quantities of pronase lost about 3,000 cpm of acid-precipitable [^{3}H]dT; this loss was measured both as a decrease in total cellular acid-precipitable [^{3}H]dT and as a quantitatively similar decrease in that contained in the Hirt supernatant fraction. A commensurate increase was measured in the culture medium; the culture medium contained a high background, presumably representing DNA specifically excreted as well as that possibly lost by dead cells. Acid-precipitable radioactivity was not lost from the Hirt Supernatant fraction when cells from the same culture were incubated with 50 μg/ml DNase I, a quantity that solubilized 98% of purified lymphocyte DNA under the same conditions. On this basis we reasoned that Hirt supernatant DNA was intracellular.

To examine the specificity of this process other proteases were tested for their capacity to induce DNA release from cells washed and resuspended in

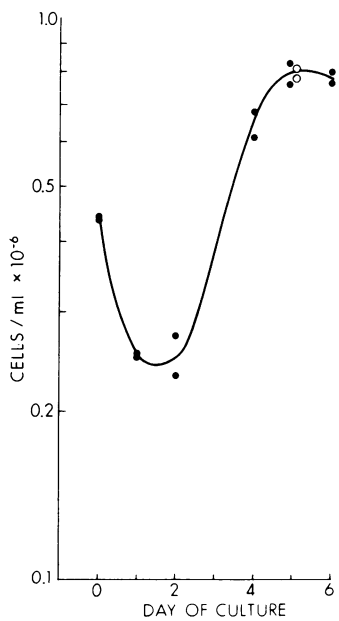

FIGURE 1 Lymphocyte growth curve. Daily cell counts were performed by the pronase-cetrimide technique (see Methods). On day 5, a second set of cells was counted after cetrimide lysis without prior exposure to pronase. ●, pronase-treated cells; ○, pronase-untreated cells.

TABLE I
Pronase-Induced DNA Release

Manipulation	DNA ([^{3}H]dT cpm)		
	Cells	Medium	Hirt supernate
Serum, *2 ml, 30 min*	22,325	5,198	4,458
0.9% NaCl, *2 ml, 30 min*	22,126	8,670	3,144
DNase I, *50 μg/ml, 30 min*	ND*	ND	3,910
Pronase, *mg/ml, 15 min*			
0.005	24,045	7,373	3,948
0.01	22,376	8,750	2,838
0.1	19,040	9,613	1,478
1.0	19,878	10,470	947
2.5	19,836	11,412	877

Quadruplicate tubes containing 2 ml of lymphocytes in complete culture medium on day 5 of culture were manipulated by the additions shown, and incubated at 37°C for designated periods. The amount of acid-precipitable [^{3}H]dT was determined in the medium (corrected to 2 ml) and Hirt supernate of two tubes from each set, and total cell acid-precipitable [^{3}H]dT was determined on the other two tubes of each set (see Methods). The mean of two determinations is shown. Serum was heterologous human serum incubated at 37°C for 2 h before use.

* Not done.

protein-free incubation medium. Trypsin was very efficient in this regard. Trypsin induced rapid accumulation of DNA in the incubation medium; as demonstrated in Fig. 2, most of this media DNA was accounted for by a reciprocal fall in Hirt supernatant DNA. The rate of DNA release was dependent upon the concentration of trypsin in the medium; as little as 1 μg/ml for 5 min caused near maximal release. Once the maximal amount was released, longer incubation or addition of more enzyme did not result in further accumulation of DNA in the incubation medium. Quantitatively, this process was reproducible. In a series of 33 separate experiments, a maximum of 13.3 ±6.9% (mean±SD, range 3.4–35%) of the total labeled DNA was released of day 5 of culture by treating cells with 1 μg/ml trypsin. In contrast to the findings for Hirt supernatant DNA, 99% of trypsin-released DNA was solubilized after incubation with soybean trypsin inhibitor and 50 μg/ml DNase I at 37°C for 30 min.

Trypsin treated with diisopropylphosphorofluoridate to inactivate its proteolytic activity (14) caused no DNA release, even when a concentration of 50 μg/ml was used. Similarly, trypsin inactivated with soybean trypsin inhibitor was completely ineffective. Plasmin at a concentration of 10 μg/ml, freshly activated from purified human plasminogen with streptokinase, also caused DNA release but required 20 min of incubation to reach the maximum amount (data not presented). Bovine and human thrombin (50 μg/ml), human Factor X_a (10 μg/ml), and neuraminidase were ineffective in causing DNA release.

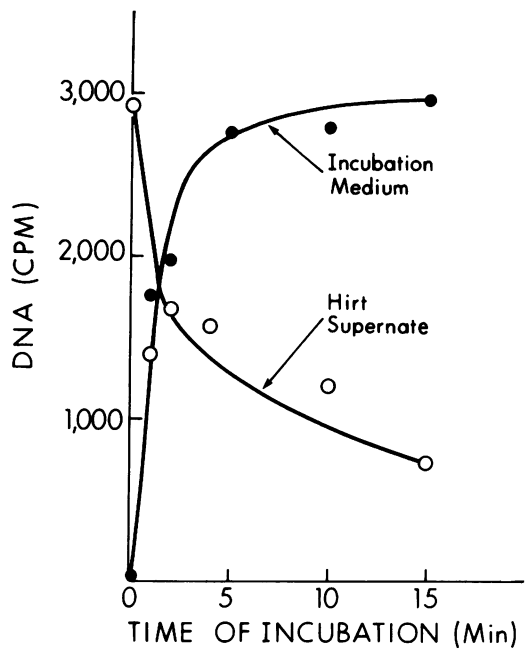

FIGURE 2 Time-course of trypsin-induced DNA release. On day 5 of culture, duplicate sets of cells resuspended in 1 ml HBSS were incubated in the presence or absence of 5 μg trypsin for the time periods shown. The incubations were terminated by adding 25 μg soybean trypsin inhibitor and chilling the suspensions to 0°C. The amount of DNA released into the incubation medium above control (●) and the amount of DNA in the Hirt supernate (○) were then determined (see Methods). Numbers presented are the mean of duplicate determinations. Total cell DNA was 20,714 cpm (not shown). For control sets incubated without trypsin, at 5 min, 644 cpm, and at 15 min, 826 cpm, were present in the incubation medium.

Different compounds that potentially could influence DNA release were screened. No DNA release was observed when lymphocytes were exposed to 25 μg/ml leukoagglutinating phytohemagglutinin, 25 μg/ml erythroagglutinating phytohemagglutinin, 12.5 μg/ml concanavalin A, 12.5 μg/ml *A. bisporus* agglutinin, 0.5 μg/ml A23187 calcium ionophore, 10^{-5} M valinomycin, 100 mM vincristine, 100 mM colchicine, or 4 mM cytocholasin B; similarly, these agents did not inhibit trypsin-induced DNA release.

When lymphocytes were incubated in the presence of 5 mM DB-cAMP for 16 h before exposure to trypsin, DNA release was inhibited. This inhibitory effect was reproducible but the absolute amount of inhibition varied with each experiment. Two representative experiments are shown in Fig. 3, which presents the quantity of DNA (expressed as acid-precipitable [^{3}H]dT in counts per minute) released from lymphocytes under various conditions. The groups within each experiment represent: (*a*) cells not exposed to DB-cAMP but treated otherwise in an identical fashion; (*b*) cells exposed for 16 h, then washed and preincubated 30 min in HBSS with the same concentration of DB-cAMP; (*c*) cells exposed 16 h, then washed and preincubated 30 min without DB-cAMP. In each experiment, exposure to DB-cAMP for 16 h resulted in significant inhibition of DNA release. In contrast, resuspending DB-cAMP-treated cells and preincubating 30 min in the absence of DB-cAMP resulted in a significant increase in the amount of DNA subsequently released by trypsin treatment, demonstrating that the effect of DB-cAMP was, in part, reversible. In general, DNA release induced by lower doses of trypsin was more sensitive to the inhibitory effects of DB-cAMP, but this varied in different experiments and was not always observed. Cell numbers were unaffected by 16 h of incubation with DB-cAMP. Incubation of cells for 16 h with 0.5 mM methyl isobutyl xanthine and for 1 h with 0.04 mM prostaglandin E_1 did not inhibit trypsin-induced DNA release and did not cause the cells to release DNA.

Divalent cation effects on DNA release. Release of DNA was greatly affected by the divalent cation concentration of the incubation medium. Lymphocytes suspended in HBSS deficient in calcium and mag-

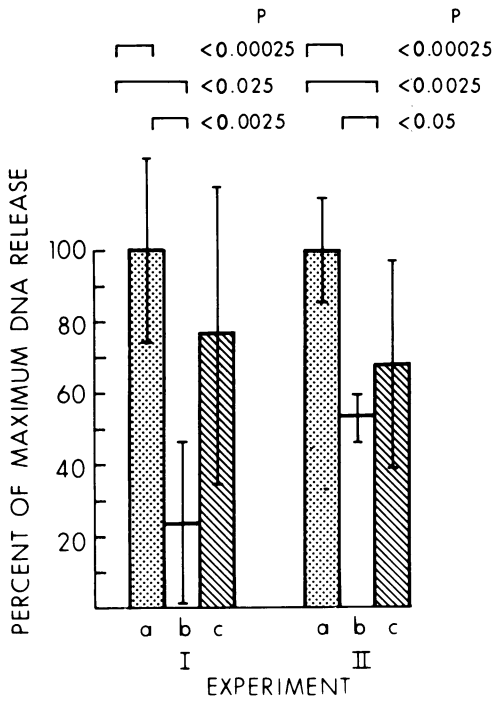

FIGURE 3 Effect of DB-cAMP on trypsin-induced DNA release. Presented are data from two different cultures, I and II. On culture day 4, one-half of a series of 2-ml lymphocyte culture suspensions was adjusted to contain 5 mM DB-cAMP. Approximately 16 h later, the DB-cAMP and control groups were each divided. One set from each group was washed and resuspended in 1 ml HBSS containing 5 mM DB-cAMP. The other set from each group was washed and resuspended in 1 ml HBSS containing no DB-cAMP. Each of the 1-ml suspensions was then layered over 0.4 ml oil and incubated for 30 min at 37°C. At the end of this preincubation time, duplicate cell suspensions from all four sets were incubated at 37°C with trypsin: 1 μg/ml for 5 min, 0.5 μg/ml for 5 and 10 min, and 0.1 μg for 15 and 30 min. Control tubes were incubated without trypsin. Incubations were stopped by spinning the cells through oil (see Methods). DNA released into the incubation medium, measured as trichloroacetic acid-precipitable [^{3}H]dT (in counts per minute), was determined by collecting precipitates on polycarbonate filters (see Methods). Data were normalized by arbitrarily assigning the mean value of counts per minute of [^{3}H]dT released from cells not exposed to DB-cAMP = 100% for any given manipulation within one experiment, and then expressing results obtained from DB-cAMP-treated cells for each different manipulation as fractions of this value. The amount of DNA released after 1 μg/ml trypsin for 5 min in experiment I = 2,300 cpm (control = 380), and in experiment II = 1,620 cpm (control = 154). Data represent the mean±SD and were compared using the null hypothesis in one-tailed Student's t test; the P values for each comparison accompany brackets above the appropriate columns. For each set, in experiment I, n = 14, and in experiment II, n = 11. The different sets are designated: (a) no overnight DB-cAMP, resuspended in HBSS; (b) overnight 5 mM DB-cAMP, resuspended in HBSS + 5 mM DB-cAMP; (c) overnight 5 mM DB-cAMP, resuspended in HBSS. The results obtained with cells exposed to DB-cAMP for only 30 min were not significantly different from those in set (a) of each experiment and, for that reason, are not graphed.

TABLE II
DNA Release in Divalent Cation-Deficient Medium

Incubation medium	Incubation time	1 ml Medium
	min	*cpm*
HBSS	5	310
HBSS plus trypsin, *1 μg/ml*	5	3,503
HBSS minus calcium	5	704
	15	1,064
HBSS minus magnesium	5	552
	15	853
HBSS minus both calcium and magnesium	5	1,839
	15	2,608

Duplicate 2-ml cell suspensions on day 5 of culture were washed with HBSS and resuspended in 1 ml of standard HBSS, or in HBSS deficient in either calcium, magnesium, or both. Standard HBSS contains 0.9 mM magnesium and 1.3 mM calcium. One set of cells resuspended in standard HBSS was treated with 1 μg/ml trypsin added at the start of the incubation periods. All sets were incubated at 37°C for the times shown. The amount of acid-precipitable [^{3}H]dT (expressed as counts per minute) was then determined for the incubation media samples and is recorded as the mean of duplicate determinations. Total cell counts per minute = 17,588.

nesium spontaneously released DNA (Table II). Ultimately, the same amount of DNA was released under these conditions as could be released by treating the cells with trypsin, and addition of trypsin to the cells after maximal spontaneous release had been achieved did not result in further release (data not presented). Suspending cells in calcium- and magnesium-free HBSS did not affect cell viability (see below). Manganese or zinc (2 mM) could substitute for magnesium or calcium in preventing spontaneous DNA release. Similar results were obtained from Tris-buffered isotonic sodium chloride was used instead of HBSS and when Tris-buffered isotonic sodium chloride was mixed with 0.27 M glucose to give a final sodium chloride concentration of 41 mM. The relative concentrations of sodium and potassium did not influence DNA release; similar results were obtained in Tris-buffered salt solutions containing 136 mM NaCl but no KCl, and in solutions containing 86 mM NaCl with 50 mM KCl.

HBSS deficient in magnesium but containing 5 mM calcium supported trypsin-induced DNA release as well as standard HBSS, but trypsin-induced DNA release was completely inhibited when lymphocytes were suspended in magnesium-free HBSS containing 10 mM calcium. This concentration of calcium did not alter the ability of trypsin to hydrolyze p-toluenesul-

fonyl L-arginine methyl ester. DNA release was similarly inhibited when lymphocytes were suspended in calcium-free HBSS containing 10 mM magnesium.

The mechanism by which 10 mM calcium might inhibit DNA release was investigated as shown in Fig. 4. Lymphocytes were incubated in magnesium-free HBSS containing 10 mM calcium for 5 min at 37°C in the presence or absence of 1 μg/ml trypsin. The action of trypsin was inhibited by the addition of soybean trypsin inhibitor, the cells were washed twice in magnesium-free HBSS containing 10 mM calcium, then were suspended in standard HBSS and incubated at 37°C. Cells not previously exposed to trypsin were treated with 1 μg/ml for 5 min and released the expected amount of DNA. Cells treated with trypsin for 5 min before washing released about 20% of the maximal amount of DNA upon resuspension in standard HBSS, but this amount did not increase significantly during the subsequent 20-min incubation. However, when trypsin was again added to these cells, the expected amount of DNA was released in 5 min, suggesting that 10 mM calcium may prevent trypsin from acting upon some structure necessary for DNA release.

Effect of buffers on lymphocyte viability. To investigate the possibility that the buffered salt solutions, particularly calcium- and magnesium-free HBSS, might damage PHA-stimulated lymphocytes, cells were washed and exposed on day 5 of culture to serum-free MEM-α, to HBSS, or to calcium- and magnesium-free HBSS for 5 min, pelleted gently, resuspended in complete culture medium, and cultured for 2 days more. As shown in Fig. 5, cell counts after this manipu-

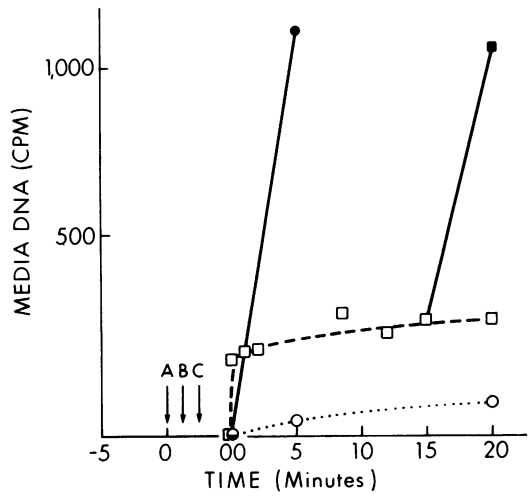

FIGURE 4 Reversible inhibition of trypsin-induced DNA release by increased calcium concentration. Lymphocytes from day 5 of culture were washed and resuspended in 1 ml magnesium-free HBSS containing 10 mM calcium. The cells were then incubated for 5 min in the presence or absence of 1 μg/ml trypsin during the period of time designated −5-0. Then 25 μg soybean trypsin inhibitor was added to each 1-ml cell suspension (point A), and the cells were washed in magnesium-free HBSS containing 10 mM calcium plus 25 μg/ml soybean trypsin inhibitor (point B). The cells were washed a second time in magnesium-free HBSS containing 10 mM calcium (point C) and finally resuspended in HBSS (point 00). At this time, cells not previously exposed to trypsin were incubated at 37°C in the presence or absence of trypsin as follows: ○, no trypsin, for 5 and 20 min; ●, 1 μg/ml trypsin, for 5 min. Cells exposed to trypsin in the −5-0 time period were incubated as follows: □, no trypsin, for periods of 0, 1, 2, 8, 12, 15, and 20 min; ■, no trypsin for 15 min, then 1 μg/ml trypsin for 5 min. Incubation periods were stopped by spinning the cells through oil (see Methods). DNA released was measured as acid-precipitable counts per minute and the values shown represent the mean of two determinations. The maximum amount of DNA released in this experiment represented 5.3% of the total labeled cell DNA.

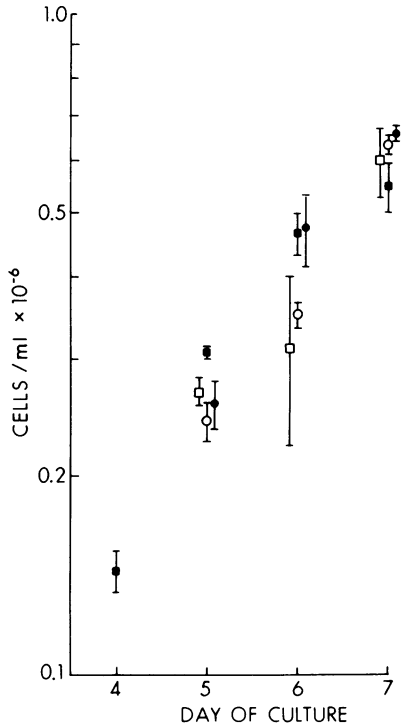

FIGURE 5 Influence of different resuspension buffers on cell number and cell growth. Replicate sets of 2-ml cell suspensions on day 5 of culture were gently pelleted and resuspended in sterile MEM-α, HBSS, or magnesium- and calcium-free HBSS. After 5 min incubation at 37°C, the cells were again gently pelleted, resuspended in complete culture medium, and returned to the incubator. Triplicate cell counts were performed by the pronase-cetrimide technique (see Methods) immediately after the manipulations on day 5 and subsequently on days 6 and 7 and were compared to triplicate cell counts performed on cells remaining in their original media and unmanipulated on days 5 of culture. Cell counts are shown as the mean±SD of triplicate determinations: ■, unmanipulated cells; □, cells incubated in MEM-α; ○, cells incubated in HBSS; ●, cells incubated in magnesium- and calcium-free HBSS.

lation were essentially the same regardless of the buffer used; these counts were about 16% lower than counts obtained on unmanipulated cells, but this is reasonably explained by unavoidable losses during media changes. Trypsin-induced DNA release in the unmanipulated cells was comparable to that observed in other experiments. Over the next 2 days, cells in all sets grew comparably. Clearly, HBSS and calcium- and magnesium-free HBSS are no more harmful to cells than the standard culture medium.

Experiments to assess specificity of DNA release: DNA release from other cell types. Two other cell types were tested to determine whether trypsin would cause DNA release in a manner similar to that occurring with PHA-stimulated human peripheral blood lymphocytes. When human diploid fibroblasts were grown in monolayer cultures in the presence of [^{3}H]dT to near confluence, exposure to trypsin caused essentially no additional DNA release over that observed in control cultures (Table III). Similarly, a long-term near-diploid human T-lymphoblast cell line, H-SB2 (15), was grown in suspension culture in the continuous presence of [^{3}H]dT. When incubated with trypsin, only 1.5% of the total 24,200 cpm incorporated into cell DNA was released into the incubation medium (Table III), compared with 13.3±6.9% released by cultured human lymphocytes under the same conditions.

Release of other cell components. Trypsin-induced DNA release was compared to release of lactic dehydrogenase, ^{51}Cr, and RNA under the same conditions. Chromium labeling and release is a standard immunologic technique used to assess cytotoxicity under a number of different conditions (16). As described in the legend to Table IV, cell samples to be used for lactic dehydrogenase and ^{51}Cr measurements were first washed, preincubated 5 min in HBSS, then resuspended in fresh HBSS; samples for DNA release in those experiments were handled in an identical fashion. The preincubation step was found to be necessary to lower the background of enzyme activity or radioactivity in the incubation medium because up to 20% of each, but no DNA, was released into the preincubation medium.

The results in Table IV demonstrate that in experiments where 7.5–12% of acid-precipitable [^{3}H]dT was released, only 1.0–4.4% of ^{51}Cr and 0–1.3% of lactic dehydrogenase were released by treatment with 1 μg/ml trypsin for 5 min. In other experiments, this amount of trypsin did not alter the activity of commercial lactic dehydrogenase under similar conditions; similarly, no inhibitor of lactic dehydrogenase activity could be demonstrated in incubation medium from cells treated with trypsin. Acid-precipitable [^{3}H]-uridine was released by trypsin treatment in amounts representing half of the amount of DNA released under identical conditions. When this trypsin-released [^{3}H]-uridine was treated with 0.1 N NaOH for 16 h at 37°C, 67% was rendered acid soluble; thus, most of the label was present in RNA. The nature of this RNA has not been investigated yet but may be a matter of some interest. Taken together, these data demonstrate a relative specificity for DNA release that would not be expected if, for instance, mild trypsin treatment simply lysed a portion of the cell population.

TABLE III
Effect of Trypsin on DNA Release by Other Cell Types

	Cell type	Trypsin	Incubation time	Total cpm present in media	Cell pellet	Total cpm present in media
		μg/ml	min		cpm	%
(a)	Diploid human fibroblasts*	0	5	913	11,408	8.0
		0	5	630	22,865	2.8
		5	5	1,021	21,649	4.8
		5	5	696	22,354	3.1
(b)	H-SB2 lymphoblasts‡	0	5	160	24,222	0.7
		0	5	165		0.6
		1	5	512		2.1
		1	5	510		2.1
		1	15	484		1.9
		1	15	400		1.6
		5	5	460		1.9
		5	5	498		2.0
		5	15	528		2.2
		5	15	542		2.2

* Diploid human fibroblasts were cultured in 75 cm^2 T-flasks containing 20 ml MEM-α, 15% fetal calf serum, and 0.25 μCi/ml [^{3}H]dT (50 mCi/mmol). Medium was changed three times per week. After 13 days in culture after reaching confluence, the cells in each flask were washed, suspended in HBSS, and transferred to 15-ml culture tubes. The final incubation volume was 1 ml. Duplicate tubes of cells were incubated in the presence or absence of 5 μg/ml trypsin for 5 min, 25 μg soybean trypsin inhibitor was added, and the amount acid-precipitable [^{3}H]dT present in the medium and the cells was determined (see Methods).

‡ Cells from the human T-lymphoblast cell line, H-SB2, were cultured in 2-ml vol, at an initial cell concentration of 10^6 cells/ml, in RPMI 1640 plus 20% fetal calf serum and 0.25 μCi/ml [^{3}H]dT (50 mCi/mmol). On day 5 of culture, the cells were washed, resuspended in 1 ml HBSS, and incubated for 5 or 15 min in duplicate in the presence or absence of 1 or 5 μg/ml trypsin. At the end of the incubation periods, five-fold excess (by weight) soybean trypsin inhibitor was added, and medium and cell acid-precipitable [^{3}H]dT was determined. The mean duplicate cell counts on the day of experiment: without pronase, 2.04×10^6 cells/ml; with pronase, 1.27×10^6 cells/ml. Total cell acid-precipitable [^{3}H]dT averaged 24,222 cpm.

TABLE IV

DNA Release Compared to Release of Other Cell Components

		Experiment number														
		1			2			3			4			5		
	Trypsin	Medium	Cell	Release	Medium	Cell	Release	Medium	Cell	Release	Medium	Cell	Release	Medium	Cell	Release
				%			%			%			%			%
DNA (^{3}H cpm)	0	292	14,394		174	17,578		440	22,552		400	24,714		286	20,690	
	+	1,976		12	1,489		7.5	2,732		10	3,064		11	2,209		9.3
LDH* activity	0	0.095	0.67		0.060	0.62			—			—			—	
	+	0.071	0.64	0	0.060	0.54	1.3									
^{51}Cr (cpm)	0	6,248	86,587		5,150	78,083		6,742	64,974			—			—	
	+	7,400	90,101	1.0	6,625	62,406	4.4	8,289	70,990	2						
RNA (^{3}H cpm)	0		—			—			—		470	14,606		889	18,919	
	+										1,147		4.6	1,687		4.2

Lymphocytes from the same culture were used for all determinations within the same experiment. Lactate dehydrogenase activity in the incubation medium and cells was determined before and after trypsin treatment as described in Methods. Cells for these experiments were washed and suspended in 1 ml HBSS and allowed to preincubate for 5 min, 37°C, then were resuspended in fresh HBSS for determination of trypsin-releasable [^{3}H]dT and lactate dehydrogenase activity. This enzyme activity is expressed as the amount of enzyme per milliliter of incubation medium or in the disrupted cell pellets causing the designated change in A 1 cm/340 per minute. In cultures used for comparison of RNA and DNA release, one set of cells was continuously labeled from day zero with 0.25 μCi/ml [^{3}H]dT (50 mCi/mmol); a second set of cells was labeled with 2.5 μCi/ml uridine-5-^{3}H (29 mCi/mmol) for 48 h from day 3 to day 5; on day 5 both sets were washed and resuspended in HBSS, and the amounts released into the medium were determined. To minimize RNase destruction of the released RNA during incubation, autoclaved solutions were used, and the trypsin was made 0.02% with respect to diethylpyrocarbonate 1 h before use. This treatment did not alter the enzyme's proteolytic activity.

* Lactate dehydrogenase.

Experiments to determine time of synthesis and intracellular location of trypsin-releasable DNA

Releasable DNA is synthesized during active cell growth. We estimated the approximate time in the culture period during which trypsin-releasable DNA was synthesized by initially starting the culture without [^{3}H]dT, then adding isotope to replicate sets of tubes on days 2, 3, 4, and 5 and measuring trypsin-releasable DNA over the subsequent days in culture. As demonstrated in Fig. 6, a significant amount of labeled DNA was released by trypsin treatment beginning on day 5, regardless of whether the isotope was added on day 2, 3, or 4. Maximum release of DNA was obtained on days 6 and 7. During this time period, the cells reached a stationary phase. In contrast, although cells in the set labeled beginning day 5 incorporated similar amounts of isotope, they released only one-third to one-half the amount of DNA compared to those cells labeled earlier, demonstrating the isotope had to be present on day 4, during active cell growth, if maximal DNA release were to be observed on subsequent culture days. This finding is more striking when the growth curve is considered (Fig. 6). Cell numbers progressively declined on days 7 and 8; if trypsin release of DNA depends upon the presence of dying cells, much more DNA should have been released on those days. In addition, this time-course of DNA release suggests that a period of 24–48 h was required before DNA synthesized on day 4 was releasable by trypsin treatment. This is consistent with previous estimates (3).

Intracellular location of DNA before release. The intracellular location of releasable DNA was assessed by separating cells into nuclear and cytoplasmic fractions before and after exposure to trypsin. Release of DNA into the medium by trypsin was accompanied by a simultaneous commensurate fall in acid-precipitable [^{3}H]dT from the nuclear fraction (Table V). Only a small amount of [^{3}H]dT was precipitable from the cytoplasmic fraction, and the amount was not altered by trypsin treatment. When exogenous ^{14}C-labeled DNA was added to the cell suspension before the separation procedure, 87% was recovered in the cytoplasmic fraction, excluding the possibility that DNA was selectively lost from this fraction. Nuclear pellets prepared by this method, when examined by electron microscopy, contained 96% nuclei that lacked the outer layer of the nuclear membrane and were free of associated identifiable cytoplasmic structures such as mitochondria. Thus, the Hirt supernatant DNA that is selectively released by trypsin treatment is associated with the nuclear cell fraction.

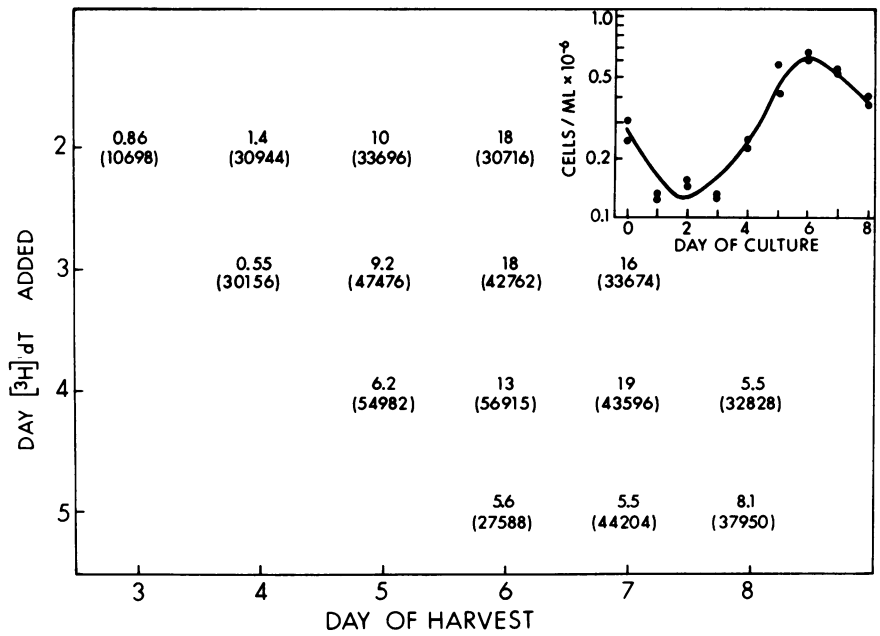

FIGURE 6 Estimation of the time of synthesis of trypsin-releasable DNA by isotopically labeling DNA for different periods in culture. Lymphocytes were cultured initially in the absence of [^{3}H]dT. Replicate sets of cells were labeled beginning on days 2, 3, 4, and 5 of culture by the addition of sterile [^{3}H]dT (50 mCi/mmol) at a concentration of 0.25 μCi/ml. Duplicate tubes of cells were harvested on the subsequent culture days by washing, resuspending in HBSS, and incubating in the presence or absence of 1 μg/ml trypsin for 5 min at 37°C. Incubations were stopped by spinning the cells through oil. Medium and cell acid-precipitable [^{3}H]dT were determined (see Methods). The numbers presented represent the mean percent of total cell counts per minute released by trypsin treatment into the incubation medium. The amount of total cell acid-precipitable [^{3}H]dT is shown in parentheses. Daily counts of cell numbers performed in duplicate are shown in the *inset*. Cell numbers on each day were essentially the same in all sets, thus only points for control cells grown in the absence of isotope are plotted.

Kinetic complexity of trypsin-released and Hirt supernatant DNA. To confirm that trypsin-released DNA represents the same sequences identified in excreted DNA, DNA released by trypsin treatment on day 5 of culture was purified, sheared by sonication, and analyzed by reassociation kinetics (2, 3) in two different sets of experiments. Trypsin-released DNA purified from a single culture reassociated in a major component representing 54% of the DNA, with a $C_0t½$ of 68±4 mol·s/liter (value at which DNA reassociation is 50% complete) (Fig. 7A). Trypsin-released DNA was subsequently purified from four consecutive cultures, pooled, and analyzed. The pooled DNA reassociated in a major component representing 54% of the DNA, with a $C_0t½$ value of 78±2.3 mol·s/liter (Fig. 9). These values are similar to the average $C_0t½$ value of 86 mol·s/liter previously identified for excreted DNA. Excreted DNA isolated in previous experiments (2, 3), which gradually accumulated in the culture medium over the period of days 3–6 in culture, was contaminated with a variable portion of cellular DNA arising from dead cells (2). These circumstances

TABLE V
DNA Is Associated with the Nuclear Fraction before Release by Trypsin

	DNA ([^{3}H]dT cpm)		
	Medium	Nuclei	Cytoplasm
No trypsin, *5 min*	432	13,290	773
	500	14,964	554
Trypsin, *5 min*	3,590	11,126	964
	3,580	10,434	940
Trypsin, *15 min*	3,070	9,040	566
	3,388	11,080	871

Duplicate 2-ml lymphocyte suspensions on day 5 of culture were washed, resuspended in 1 ml HBSS, and incubated at 37°C for 5 or 15 min in the presence or absence of 1 μg/ml trypsin. Incubations were stopped by adding 5 μg soybean trypsin inhibitor and chilling the suspensions to 0°C. The amount of acid-precipitable [^{3}H]dT was determined in duplicate for the incubation medium and for nuclear and cytoplasmic fractions prepared as described (see Methods).

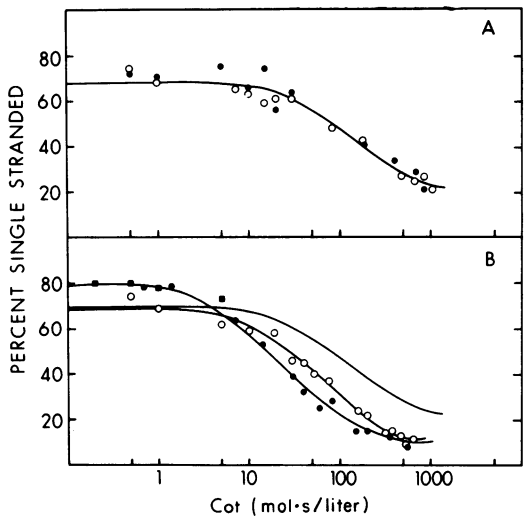

FIGURE 7 Reassociation analysis of trypsin-released DNA isolated from the incubation medium. (A) In two separate experiments incubation medium DNA, 13.8 μg in 15.0 μl (○), and 13.8 μg in 15.2 μl (●), reassociated in a major component comprising 54% of the DNA with a $C_0t½$ of 68±4 mol·s/liter, RMS = 3.8%; 17% of the DNA remained unreassociated within the C_0t ranges studied. Data points were analyzed and plotted as previously described (2, 3). (B) The reassociation of incubation medium DNA was driven by a 10-fold excess of total cell DNA from resting lymphocytes (○) or the high molecular weight Hirt pellet fraction of cell DNA from cultured lymphocytes, harvested on day 3 (●, ■); the curve for medium DNA above is included for reference. C_0t values were calculated on the basis of the concentration of medium DNA alone (2, 3). In one experiment, 11.5 μg medium DNA in the presence of 115 μg of resting lymphocyte DNA in 17.1 μl (○) reassociated in a major component comprising 66% of the DNA with A $C_0t½$ of 43±1.7 mol·s/liter, RMS = 2.7%; 4% remained unreassociated. In two experiments, 11.5 μg medium DNA plus 115 μg day 3 Hirt pellet DNA in 15.0 μl (●), and DNA in 22.2 μl (■), reassociated in a major component comprising 73% of the DNA with a $C_0t½$ of 14.1±0.7 mol·s/liter, RMS = 2.7%; 9% remained unassociated.

may account for the range of $C_0t½$ values previously observed. In the present experiments, DNA was specifically released from washed cells and should more accurately reflect the true complexity of excreted DNA sequences.

In the presence of a 10-fold excess of unlabeled, sheared resting lymphocyte total cell DNA, the reassociation of this major component was accelerated by a factor 0.8 times that expected if sequences comprising the major component were present once per haploid genome (Fig. 7B). The basis for these calculations is detailed by Rogers (3). In the presence of sheared Hirt pellet DNA isolated from cells on day 3 of culture, the reassociation of this major component was accelerated by a factor 2.3 times that expected for sequences present once per haploid genome (Fig. 7B). Thus, on day 3, high molecular weight cell DNA contained about threefold more sequences similar to trypsin-released DNA than did resting lymphocyte DNA. As discussed in detail previously (3), reassociation analysis of these complex DNA populations provides only reasonable approximations of sequence numbers. However, these results are consistent with previous estimates of numbers of copies of excreted DNA sequences under similar circumstances (3).

Hirt supernatant DNA isolated from day-5 cells from different cultures reproducibly contained a component representing 34% of the DNA that reassociated with a $C_0t½$ of 26±0.9 mol·s/liter (Fig. 8A). Presumably, the 37% remaining unreassociated in these experiments represented cellular DNA of high complexity. As illustrated in Fig. 8B, after trypsin treatment, DNA remaining in the Hirt supernate had lost this lower complexity component and reassociated in a major component representing 65% of the DNA with a $C_0t½$ of 448±30 mol·s/liter. It is unlikely that data scatter accounts for the difference between the two Hirt supernatant DNA preparations because the curves are derived from three (Fig. 8A) and two (Fig. 8B) separate reassociation experiments, respectively. However, this possibility was further assessed by providing the computer with the data points in Fig. 8A (for Hirt supernatant DNA before trypsin release) and the best solution giving the curve in Fig. 8B (for Hirt supernatant DNA after trypsin release); the root mean square (RMS) for this solution was 6.2%, a value much higher than that obtained for the best fit drawn in Fig. 8A, 3.2%. Similarly, the data points from Fig. 8B, when fit with the solution for the curve in Fig. 8A, give an RMS = 6.1%, a value again higher than that obtained for the best fit drawn in Fig. 8B (4.8%). It is difficult to base a statistical probability on these comparisons. For that reason we further tested our hypothesis that trypsin treatment depletes Hirt supernatant DNA of sequences identified as the major component in released DNA. On day 5 of culture, half of the cells were treated with 1 μg/ml trypsin in the standard manner; Hirt supernatant DNA was then purified separately from untreated and from trypsin-treated cells and used to drive the reassociation of pooled trypsin-released DNA to determine the relative proportions of similar sequences in the two preparations. As shown in Fig. 9, in the presence of a five-fold excess of Hirt supernatant DNA from untreated cells, the major component of trypsin-released DNA reassociated 3.0 times faster than control, whereas a five-fold excess of Hirt supernatant DNA from trypsin-treated cells accelerated its reassociation by a factor of 1.6. Thus, Hirt supernatant DNA from trypsin-treated cells contained approximately half the quantity of sequences similar to trypsin-released DNA when compared with untreated cells.

In these experiments, the addition of Hirt supernatant DNA doubled the amount of trypsin-released DNA reassociating by a C_0t value (concentration of

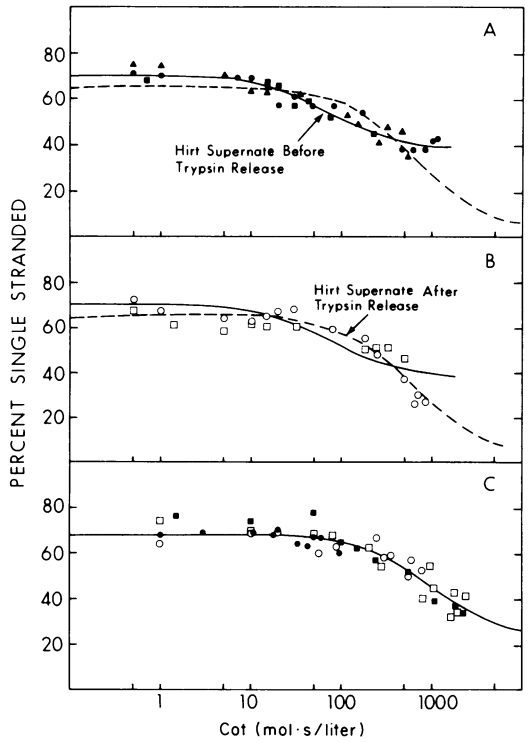

FIGURE 8 Reassociation analysis of Hirt supernatant DNA. (A) Hirt supernatant DNA was isolated on day 5 of culture from PHA-stimulated lymphocytes that were not treated with trypsin. This DNA was studied in three experiments: 12.0 μg DNA in 20.1 μl (●), 12.0 μg DNA in 15.0 μl (■), and 14.5 μg DNA in 16.3 μl (▲) reassociated in a major component comprising 34% of the DNA with a $C_0t½$ of 26 ±0.9 mol·s/liter, RMS = 3.2%; 37% remained unreassociated. The curve for Hirt supernatant DNA isolated from cells after trypsin release is included as a reference (data presented in B). (B) Hirt supernatant DNA was isolated on day 5 from the same culture as in A but from cells that were first treated with 1 μg/ml trypsin for 5 min. This DNA was studied in two experiments: 14.2 μg DNA in 15.0 μl (○), 6.4 μg DNA in 19.1 μl (□) reassociated in a major component comprising 65% of the DNA with a $C_0t½$ of 448 ±30 mol·s/liter, RMS = 4.8; 0.7% remained unreassociated. The curve for Hirt supernatant DNA isolated from cells without prior trypsin treatment is included as a reference (data presented in A). (C) DNA from resting lymphocytes freshly isolated from 3 liters of human blood was separated into Hirt supernatant and Hirt pellet fractions, and purified. Samples of 0.5 μg, in 100 μl reaction mixtures, were labeled with [^{3}H]dT by nick translation without DNase as described by Maniatis et al. (18); final specific activities for each were approximately 5×10^7 dpm/μg. Both labeled and unlabeled Hirt supernatant and pellet DNAs were sheared by sonication (2), and analyzed by reassociation kinetics after mixing 75,000 cpm of the labeled DNA with the stated amount of the appropriate unlabeled DNA. Resting lymphocyte Hirt supernatant DNA, 20.5 μg in 21 μl (●), and 20.5 μg in 18.4 μl (■), and resting lymphocyte Hirt pellet DNA, 54.9 μg in 22.6 μl (○), and 54.9 μg in 15.9 μl (□) reassociated in a major component comprising 47% of the DNA with a $C_0t½$ of 470±23 mol·s/liter, RMS = 4.5%. 22% remained unreassociated. Because the reassociation curves of the two DNAs, supernate and pellet, could not be distinguished as different, they are plotted and analyzed together in this figure.

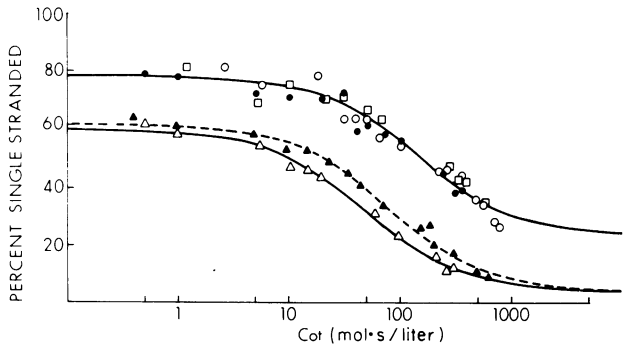

FIGURE 9 Reassociation analysis of trypsin-released DNA pooled from four consecutive cultures: effect of excess Hirt supernatant DNA. Pooled trypsin-released DNA was studied in three reassociation experiments: 18.9 μg in 19.4 μl (○), and 18.9 μg plus 189 μg salmon sperm DNA in 17.1 μl (●) and in 15.0 μl (□) reassociated in a major component comprising 54% of the DNA with a $C_0t½$ of 78±2 mol·s/liter, RMS = 3.2%; 24% remained unreassociated. The reassociation of this pooled trypsin-released DNA was then studied in the presence of a fivefold excess of unlabeled Hirt supernatant DNA obtained from lymphocytes on day 5 of culture. Before isolation of Hirt supernatant DNA, cells from this culture were divided into two sets; one set was not treated with trypsin (△ —— △), while the other was treated with 1 μg/ml trypsin in the standard manner (▲ - - - ▲). Pooled trypsin-released DNA, 18.9 μg, plus 94.5 μg of Hirt supernatant DNA from untreated cells in 20 μl (△) reassociated in a major component comprising 55% of the DNA with a $C_0t½$ of 25.7±0.8 mol·s/liter, RMS = 1.6%; 4.6% remained unreassociated. In contrast, pooled trypsin-released DNA, 18.9 μg, plus 94.3 μg Hirt supernatant DNA from trypsin-treated cells in 27.8 μl (▲) reassociated in a major component comprising 58% of the DNA with a $C_0t½$ of 48.9±1.5 mol·s/liter, RMS = 1.9%; 3.6% remained unreassociated.

nucleotides·time) of 1 M·s/liter (from 20 to 40%), and decreased the terminal unreassociated fraction by a similar percentage. In other experiments, we have observed that this change is proportional to the quantity of repetitive sequence elements added; under these circumstances the fraction of DNA in the major component, previously characterized as representing unique sequence elements (2, 3), is not changed.[2] These terminal unreassociated sequences in trypsin-released DNA are of considerable interest; their nature and organization are presently under study. However, their presence should not alter the conclusion derived from the experiment presented in Fig. 9.

We conclude that trypsin treatment released from the Hirt supernatant fraction of cell DNA those sequences previously identified as excreted DNA (2, 3) which are composed of unique sequence elements having a complexity of about 10% of the genome, sequences that are amplified during cell growth.

Although all of the evidence presented so far suggests that trypsin releasable or excreted DNA sequences must be newly synthesized, other authors

[2] Rogers, J. Work in progress.

have claimed that resting lymphocytes specifically release DNA (17). To assess whether trypsin-releasable or excreted DNA sequences might be present in resting lymphocytes in quantities too small to have been detected in other experiments, we purified Hirt supernatant and Hirt pellet DNA from lymphocytes obtained from 3 liters of fresh human blood; of the 6.8 mg of DNA obtained, 320 μg (4.7%) represented Hirt supernatant DNA. Both supernatant and pellet DNAs were labeled with [^{3}H]dT by the nick translation method of Maniatis et al. (18), sheared, and analyzed by reassociation kinetics. As demonstrated in Fig. 8C, DNA from the two fractions reassociated in a similar manner; we could not identify a difference between resting lymphocyte Hirt supernatant and Hirt pellet DNA within the limits of sensitivity of this method.

DISCUSSION

In previous studies (2, 3), our culture system utilized higher cell densities and provided less than optimal cell growth. Excreted DNA was isolated from these cultures after it gradually accumulated in the medium over a period of 3 days. Under these circumstances both the effect of DNA that might be released by dying cells and the effect of nucleases in the culture medium would make quantitation of excreted DNA relative to total cell DNA difficult. It was possible to measure synthesis of extra copies of excreted DNA sequences by reassociation analysis (3). These results suggested that on day 3 or 4 of culture as much as 30% of cellular DNA might represent amplified excreted DNA sequences. The work presented here provides another means of estimating the quantity of extra DNA involved. This information is pertinent to the general field of lymphocyte physiology because most assays of lymphocyte "proliferation" utilize cultures with high cell densities and only measure incorporation of [^{3}H]dT into acid-precipitable form. Thus, it is possible that a significant amount of label is incorporated into excreted DNA. The culture and cell counting techniques developed by Stewart and coworkers (8, 9) assess true proliferation; we chose to use their system in order to better estimate the amount of excreted DNA synthesized in rapidly proliferating cells.

The results presented here establish that synthesis of excreted sequences occurs during logarithmic growth, that the immediate precursor to excreted DNA released from lymphocytes can be identified in the Hirt supernatant fraction of cell DNA, and that excreted DNA sequences are selectively released from this fraction when lymphocytes are exposed for brief periods of time to small quantities of different proteases, 1 μg/ml trypsin for 5 min being optimal. The maximum amount of DNA released by trypsin treatment varied little from culture to culture and constituted about 13% of the newly synthesized DNA. Excreted DNA is released spontaneously with time in culture (1–3); because our selection of day 5 for trypsin treatment ignores any DNA released earlier, 13% probably represents a low estimate of the actual amount of excreted DNA synthesized.

The release was specific as shown by a failure of other components such as lactic dehydrogenase, ^{51}Cr, or RNA to be released to a comparable degree, and by the fact that little or no DNA was released from human diploid fibroblasts or H-SB2 lymphoblasts treated in an identical manner. Trypsin-induced DNA release appeared to be an active process as judged by the reversible inhibition of release occurring after prolonged exposure to DB-cAMP. Under our incubation conditions, divalent cations were found to be important in influencing release; in the absence of divalent cations DNA was released spontaneously, whereas increased concentrations (10 mM calcium or magnesium) completely inhibited trypsin-induced release.

Our experiments utilizing NP-40 lysis to fractionate cells into nuclear and cytoplasmic fractions indicated that trypsin-releasable Hirt supernatant DNA was associated with the nuclear fraction. This result was unexpected because it differed from that obtained in a somewhat similar system. Lerner et al. (19) used a similar procedure to obtain cytoplasmic membrane-associated DNA from a long-term lymphoblast cell line; the unique sequence components of cytoplasmic membrane-associated DNA and of excreted DNA are of the same order of complexity (2, 20) but have not been compared directly. The nuclear association of excreted DNA before release requires a mechanism capable of transporting it through a substantial intracellular distance in a brief period of time. There is presently no information about the nature of this mechanism, and we are not aware of other reports regarding similar movement of nucleic acids. However, there are well-studied secretory systems in which large numbers of cellular organelles are rapidly transported to the cell periphery after an external stimulus. For example, blood platelets release the contents of their cytoplasmic granules within seconds after treatment with the serine protease thrombin (21), whereas anti-IgE antibody or concanavalin A can induce degranulation of tissue mast cells within a period of several minutes (22).

The mechanism by which trypsin induces DNA release is unexplained but requires that the enzyme be active. The fact that cells exposed to trypsin in the presence of 10 mM calcium released little DNA when washed free of enzyme and resuspended in standard incubation buffer, but then were able to release the maximal amount of DNA when again exposed to the enzyme, suggests that there may be a membrane struc-

ture responsible for triggering DNA release that is accessible to trypsin under standard conditions but protected at increased calcium concentrations. Serine proteases are widely distributed among pathways for blood coagulation, complement activation, and kinin generation; it is possible that one of these many proteases could act during the immune response to trigger DNA release in a similar manner in vivo, although at present the synthesis of excreted DNA sequences has been demonstrated only in vitro.

Other authors have reported DNA release by lymphocytes (17, 23, 24) but the different systems utilized in those reports make direct comparisons difficult. It is of interest that Boldt et al. (24) labeled purified populations of lectin-stimulated human B and T lymphocytes with [^{3}H]dT and demonstrated that the same relative proportion of newly synthesized DNA was excreted from each cell type. However, the possibility that this phenomenon may be limited to a specific subpopulation of lymphocytes still has not been resolved. There is still no direct evidence that synthesis and release of excreted DNA sequences is functionally important. Perhaps the reproducibility of the system described here may permit future studies to correlate this phenomenon with other parameters in the immune response, and in that way test the hypothesis that excreted DNA plays some role in normal lymphocyte physiology.

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