CHARACTERIZATION OF DNA EXCRETED FROM
PHYTOHEMAGGLUTININ-STIMULATED LYMPHOCYTES*

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Human peripheral blood lymphocytes ordinarily are resting cells, having a very low level of "spontaneous" DNA synthesis. When they are cultured in the presence of certain plant lectins or in the presence of an antigen to which the cell donor is sensitized, the cells can be stimulated to make DNA. Plant lectins, such as phytohemagglutinin (PHA), "nonspecifically" stimulate more than 80% of the lymphocytes in culture; the resultant peak of DNA synthesis as measured by uptake of radioactive precursor into acid-precipitable form occurs 3-4 days after stimulation (1). In contrast, a specific antigen is thought to stimulate relatively few cells initially; however, by the time of peak DNA synthesis, between the 5th and 8th day after stimulation, as many as 60% of the cells present may be transformed and are presumed to be synthesizing DNA (2).

Although both PHA- and antigen-stimulated lymphocyte cultures demonstrate an increased rate of mitosis, the number of cells synthesizing DNA seems to be greater than the number of cells potentially dividing (3). In investigating this discrepancy, we demonstrated that 35-90% of the DNA labeled at the peak of DNA synthesis by a 4-h pulse of [3H]thymidine was excreted from the cells during the 3 days after the pulse (3). The excreted DNA could be recovered in the culture medium. In double-label experiments utilizing both [3H]thymidine and [14C]uridine, labeled RNA was not lost from the cells, suggesting that cell lysis could not explain excretion of labeled DNA (3). However, we could not rule out the possibility that RNA might be degraded rapidly in the culture medium with the labeled nucleotides being quickly taken up again by living cells. A more definitive answer to the question of whether DNA "excretion" is a specific process of activated lymphocytes required study of the excreted DNA itself. If excreted DNA results from cell lysis or from an artifact-induced culture condition which prevent completion of mitosis, there should be little difference between excreted and total cell DNA. Alternative possible explanations for DNA excretion include activation of a latent virus or some type of DNA exchange between lymphocytes for the purpose of intercellular transmission of information. Accordingly, DNA excreted from PHA-stimulated human peripheral blood lymphocytes has been purified and characterized. I have compared excreted DNA to total lymphocyte cell DNA with respect to $T_m$ (thermal dena-
turation temperature), to thermal elution from hydroxyapatite (HAP),¹ and to its kinetics of reassociation after denaturation.

Materials and Methods

**Lymphocyte Culture.** All experiments were performed on cultures of human peripheral blood lymphocytes purified from defibrinated blood by dextran sedimentation followed by centrifugation on a Hypaque-Ficoll cushion as previously described (3). The resultant cell population contained greater than 90% small lymphocytes, the remainder of the cells being erythrocytes, neutrophils, and monocytes. Previous work (3) has shown that DNA excretion occurs in pure lymphocyte cultures and cannot be explained by contaminating populations of different cell types. Lymphocytes were cultured in disposable plastic culture tubes in 2 ml of complete medium consisting of Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 200 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 15% autologous donor serum and made 0.01 M with respect to HEPES (Sigma Chemical Co., St. Louis, Mo.) pH 7.25 to which was added a sterile solution of either \([\text{methyl-}^{14}\text{C}]\text{thymidine (50 mCi/mM)}\) or \([\text{methyl-}^3\text{H}]\text{thymidine (2 Ci/mM diluted to 50 mCi/mM with cold thymidine)}\) (both isotopes from New England Nuclear, Boston, Mass.) at a concentration of 0.25 μCi/ml. Cultures were maintained in a 5% CO₂ atmosphere at 37°C.

*Phaseolus vulgaris* E(erythroagglutinating)-PHA was prepared from Difco PHA-P according to the method of Weber et al. (4), and cultures were stimulated by adding 7 μg E-PHA to each culture tube on day zero at the start of the culture. On day 3 the cultures were centrifuged at 300 g for 10 min, the supernate was aspirated from the cell pellet, and the cells resuspended in 2 ml of fresh complete medium containing the appropriate isotope and cultured for 3 more days in the absence of PHA. In several experiments duplicate tubes were removed each day, and cell numbers were determined by vigorously suspending the cells in a cetrimide solution (5) and then counting the nuclei in a hemocytometer. On day 6 the cultures were harvested by centrifuging at 1,000 g for 10 min and carefully removing the culture medium from the cell pellets. Acid precipitable radioactivity in the cell pellets and media was determined as described previously (3), except that NCS (Amersham/Searle Corp., Arlington Heights, Ill.) digests of the precipitated material were counted in a PPO-POPOP-toluene scintillation cocktail.

**Isolation of DNA.** To purify DNA from an individual culture, cells from 100 tubes were pooled, washed twice with 0.9% NaCl, and then dispersed in 25 ml 0.01 Tris pH 7.5, 0.2 M NaCl, 0.01 M EDTA, 0.6% sodium dodecyl sulfate (Sigma Chemical Co.). Pronase (protease type VI, Sigma Chemical Co.), self digested at 37°C for 1 h, was added to a concentration of 0.5 mg/ml, and the solution was incubated at 37°C for 2 more h, extracted twice with an equal volume of chloroform:isoamyl alcohol 24:1, and precipitated with 2 vol of ethanol at -20°C. The precipitate was redissolved in 10 ml of 0.01 M Tris-HCl pH 7.5 and 0.5% sodium dodecyl sulfate, incubated at 37°C, then extracted three times with chloroform:isoamyl alcohol, and finally sonicated at 4°C with a Biosonic IV sonicator (VMR Scientific Div., UNIVAR, San Francisco, Calif.) using a 3/8 inch diameter probe tip at 60 W output for 10-s intervals alternating with 10-s rest periods for a total sonication time of 5 min. The DNA was then dialyzed at 4°C against a solution of 0.1 SSC (SSC is 0.15 M NaCl-0.015 M Na citrate)-0.25% sodium dodecyl sulfate and precipitated at -20°C for 16 h with 2 vol of ethanol and 0.1 vol 0.2 M sodium acetate pH 4.5. The precipitate was centrifuged at 4°C at 20,000 g for 20 min, washed with 100% ethanol and then with 10% ether, dissolved in 1 mM EDTA pH 7.0, and stored at 4°C over a drop of chloroform. To purify media DNA, approximately 200 ml of pooled medium from an individual culture was adjusted to 0.01 M with respect to Tris-HCl pH 7.5 and 0.5% with respect to sodium dodecyl sulfate, incubated at 37°C for 2 h in the presence of 0.5 mg/ml self-digested pronase, and extracted twice with an equal volume of chloroform:isoamyl alcohol. The pooled chloroform:isoamyl alcohol was re-extracted with 0.25 vol of 0.01 M Tris-HCl pH 7.5-0.5% sodium dodecyl sulfate; the aqueous portions were then pooled and precipitated at -20°C with 2 vol of ethanol and 0.1 vol sodium acetate pH 4.5. The precipitate was collected by centrifugation, dissolved in 30 ml of 0.14 M NaPO₄, pH 6.8-8 M urea, and applied at room temperature to a column

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¹ Abbreviations used in this paper: HAP, hydroxyapatite; PHA, phytohemagglutinin; RMS, root mean square; SSC, 0.15 M NaCl-0.015 M Na citrate.
containing 10 ml packed volume of HAP (DNA grade HTP, Bio-Rad Laboratories, Richmond, Calif.). The column was washed with 20 vol of 0.24 M NaPO₄ pH 6.8-8 M urea; DNA was eluted from the column with 0.4 M NaPO₄ pH 6.8-8 M urea. 6-ml fractions of the effluent were collected, and fractions containing radioactivity were pooled, dialyzed extensively against 0.1 SSC-0.25% sodium dodecyl sulfate, concentrated by dialysis against powdered polyethylene glycol (Carbowax 5000, Union Carbide Corp., New York), dialyzed again against 0.1 SSC-0.25% sodium dodecyl sulfate, then precipitated with 3 vol of ethanol and 0.1 vol Na acetate pH 4.5 overnight at 20°C. The precipitate was collected by centrifugation, washed with ethanol and then ether, dissolved in 0.5-1 ml of 1 mM EDTA pH 7.0, and stored at 4°C over a drop of chloroform. Recovery of media DNA judged by acid precipitable radioactivity was 75-100%. Purity of all DNA preparations was monitored by scanning their UV absorption spectra from 300-220 nM in a Beckman M-IV Acta spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.). Preparations with a 260/230 or 260/260 ratio of less than 1.8 were extracted again with chloroform:isoamyl alcohol, reapplied to HAP and washed, eluted, and precipitated as above. The concentration of purified DNA was calculated from its absorbance assuming A₂₆₀ m, 50 μg/ml = 1.0.

Tₐ Analysis. Optical Tₐ determinations were performed on 200-μl samples of DNA in SSC covered with silicone oil (no. 200 fluid, Dow Corning Corp., Midland, Mich.) in Teflon-stoppered cuvettes in a Beckman M-IV Acta double beam spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.). Preparations with a 260/230 or 260/280 ratio of less than 1.8 were extracted again with chloroform:isoamyl alcohol, reapplied to HAP and washed, eluted, and precipitated as above. The concentration of purified DNA was calculated from its absorbance assuming A₂₆₀ m, 50 μg/ml = 1.0.

Tₐ Analysis. Optical Tₐ determinations were performed on 200-μl samples of DNA in SSC covered with silicone oil (no. 200 fluid, Dow Corning Corp., Midland, Mich.) in Teflon-stoppered cuvettes in a Beckman M-IV Acta spectrophotometer equipped with a Braun circulating heat-temperature programmer permitting a linear temperature increase of 1°C/min. Temperature was monitored by a probe inside the blank cuvette. Thermal elution from HAP was accomplished by applying a DNA sample in 0.12 M NaPO₄ pH 6.8 to 1-ml packed volume of HAP equilibrated with 0.12 M NaPO₄ pH 6.8 in a water-jacketed column at 60°C. The HAP was washed with 5 vol 0.12 M NaPO₄ pH 6.8 and the effluent collected. The temperature of the column was then raised at 5°C increments up to 100°C; each time after the desired temperature had been reached the HAP was washed with 5 vol of 0.12 M NaPO₄ pH 6.8 equilibrated at column temperature. DNA eluted at each step was precipitated at 4°C in the presence of 10 μg bovine serum albumin carrier with 1 ml 100% wt:vol trichloroacetic acid and collected on Gelman GA-8 filters. For 14C-labeled DNA, the filters after drying could be counted directly in 10 ml PPO-POPOP-toluene scintillation cocktail. For 3H-labeled DNA, 0.5 ml Nuclear-Chicago (Nuclear-Chicago Co., Des Plaines, Ill.) solubilizer was added to the filters in the scintillation cocktail, the mixture was allowed to sit overnight at room temperature, then the samples were counted after adding five drops glacial acetic acid to diminish chemiluminescence.

Rate Zonal Sedimentation. Sucrose gradient rate zonal sedimentation analysis was carried out either under alkaline (sucrose in 0.1 M NaOH) or neutral (sucrose in SSC) conditions. Isookinetic gradients were poured in 5 ml polyallomer tubes by the method of Noll (6), with Cₛ = 5.0% (wt:vol), Vₛ = 5.63 ml, Cₑ = 27.8%, and Vₑ = 5.0 ml. Gradients were centrifuged in the Beckman SW 50.1 rotor at 20°C, 48,000 rpm for varying time periods, and fractions were collected from the bottom of tubes after puncturing them with a 25-gauge needle. 32P-labeled SV40 DNA II used as a gradient marker was the generous gift of Dr. L. Gelb.

Reassociation of DNA. Analysis of reassociation kinetics (Concentration of DNA × time [Cᵗ] curve analysis) was carried out in general by the methods of Britten and Kohne (7) and Britten et al. (8). Since only relatively small quantities of excreted DNA could be recovered (see results) it was necessary to concentrate this DNA and work with very small volumes. Routinely, precise volumes of DNA solutions in 1 mM EDTA were concentrated in siliconized conical 0.2-ml glass vials (Reactivials, Pierce Chemical Co., Rockford, Ill.) by evaporation under a gentle stream of dry air at room temperature to a volume of about 15 μl. The solutions were gently mixed on a vortex mixer to ensure that no DNA remained on the vial walls. In pilot experiments, greater than 95% of the radioactivity present in dilute samples was recovered when concentrated samples were transferred to scintillation vials and counted. Final volumes were subsequently calculated by removing 0.5-μl aliquots from concentrated samples with a 1 μl Hamilton syringe and counting them, assuming 100% recovery of radioactivity initially present. Reactivials containing concentrated samples were completely filled with silicone oil (no. 200 fluid, Dow Corning Corp.), tightly sealed, heated at 100°C for 5 min, and quenched immediately in an ethanol-ice slurry. The

\[^{2}C_{m}\text{, concentration of sucrose in mixing chamber; } V_{m}\text{, volume in mixing chamber; } C_{r}\text{, concentration of sucrose in reservoir; } V_{r}\text{, volume in reservoir.}\]
solutions were adjusted to 0.4 M with respect to NaPO₄ pH 6.8 by adding an appropriate amount of 4.0 M NaPO₄ pH 6.8, mixed thoroughly with a small triangular magnetic stirring bar and, after removing a zero time sample, incubated at 68°C. 0.5-μl aliquots were removed through a valved cap at appropriate intervals and diluted immediately into 1.0-ml volumes of 0.12 M NaPO₄ pH 6.8-0.4% sodium dodecyl sulfate. A sample was processed by applying it to a 1-ml-packed volume column of HAP at 60°C, washing the column with 5 vol of 0.12 M NaPO₄ pH 6.8 to elute single-stranded DNA, then washing with 5 vol of 0.4 M NaPO₄ pH 6.8-0.4% sodium dodecyl sulfate to elute double-stranded DNA. DNA in each fraction was precipitated with trichloroacetic acid at 4°C in the presence of 10 μg bovine serum albumin carrier, collected on a Gelman membrane filter, and counted as described above. The DNA was quantitatively eluted from HAP under these conditions as assessed by dissolving samples of HAP in 1 N HCl and counting them in 10 ml of Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.). Results were plotted as percent single stranded DNA vs. Cot (where Cot = A₂₆₀ × h/2 (7)). Curves were analyzed by the least-squares method using the "Finger" program of Britten (8) adapted to an IBM-360 computer. The root mean square (RMS) for each solution is included; this is a measure of the dispersion of observed points around the fitted curve (a standard deviation for the points) and has the same dimension as the data ordinate, namely percent. In addition, the standard error for the rate constant (K) estimate, sₓ, was determined for each solution. This estimate of the asymptotic variance of K was derived by maximum likelihood assuming normal residuals with constant variance by Dr. R. Wette of the Division of Biostatistics of this institution:

\[ sₓ = \text{RMS}/\sqrt{\sum_{i=1}^{n} (C_{i}C_{t}/(1 + K C_{t})^2)^{2}} \]

A derivation of the formula is available on request.

Results

Recovery of Excreted and Cell DNA. I found that PHA-stimulated cultures generally showed a twofold increase in cell number over the 6-day culture period (data not shown). This is an improvement over methods previously used (3) and may reflect a buffer combination more carefully controlling pH as well as use of smaller amounts of PHA. In experiments using this system in which lymphocytes were cultured in the absence of isotope, then pulse labeled with [³H]- or [¹⁴C]thymidine for 4 h on day 3, washed, resuspended, and cultured for 3 more days in the absence of isotope, results similar to those previously reported (3) were obtained. Routinely 30-60% of the acid-precipitable radioactivity present in the cells after labeling on day 3 was excreted from the cells and could be recovered from the culture medium on day 6. This demonstrated that DNA excretion occurs under conditions permitting active cell growth. In subsequent experiments lymphocytes were cultured with radioactive thymidine present throughout the culture period (see Materials and Methods) to achieve approximately uniform labeling of all DNA synthesized; under these conditions the quantity of acid soluble isotope in the medium at the end of the culture period was always more than 80% of that initially present.

In Table I, the results of four consecutive cultures from different donors are shown. The total amount of DNA present in cells and in the culture media in each experiment was calculated from the specific activities of the purified DNAs and from the total amount of acid-precipitable radioactivity recovered in each fraction. In some cultures (A, B, and C), the specific activity of media DNA was higher than that of DNA remaining in the cells, the media/cell ratio ranging
TABLE I

| Culture | Start of culture cells per tube × 10⁶ | Total dpm incorporated at end of culture | Total DNA at end of culture | Specific activity of DNA |
|---------|--------------------------------------|------------------------------------------|----------------------------|-------------------------|
|         |                                      |                                          |                            |                         |
|         |                                      |                                          |                            | µg                      |
|         |                                      |                                          |                            | dpm/µg                  |
| A       | 2.8                                  | ND*                                      | ND                         | 10,531                  |
| B       | 2.0                                  | 184,087                                  | 45,069                     | 9,220                   |
| C       | 1.5                                  | 220,476                                  | 43,250                     | 13,224                  |
| D       | 3.8                                  | 69,102                                   | 13,875                     | 7,494                   |

*ND, not done.

Lymphocytes were cultured as described in Materials and Methods; DNA was labeled in A and B with [³H]thymidine and in C and D with [¹⁴C]thymidine.

![Graphs](image)

FIG. 1. Media and cell DNAs analyzed by rate zonal sucrose gradients: Gradients were poured as described in Materials and Methods. (a, left) Media DNA, culture C from Table I, and (b, center) sheared cell DNA from the same culture were centrifuged on an alkaline gradient in the presence of [³²P]-SV40 DNA II marker for 5½ h at 20°C, 48,000 rpm in the Beckman SW50.1 rotor. (c, right) Media DNA was centrifuged on a neutral gradient with marker DNA for 3 h at 20°C, 48,000 rpm in the SW50.1 rotor. [³²P] (●), [¹⁴C] (○).

from 1.3–2.3. In other cultures (D), the opposite was true. The percentage of total DNA in the culture recovered from the media ranged from 9.5–21.4%.

Physical Properties of Excreted DNA. DNA was purified from culture media using a modification of the urea-HAP technique of Britten et al. (8) which retains only double-stranded DNA. Essentially complete recovery of acid-precipitable radioactivity was achieved demonstrating that little single-stranded DNA was present.

When analyzed on isokinetic (6) alkaline sucrose gradients, media DNA sedimented with a peak between 7–8S (Fig. 1a), when compared to SV40 DNA II composed under alkaline conditions of single-stranded 18S circular and 16S linear molecules (9). Similar results were obtained on neutral sucrose gradients (Fig. 1c); SV40 DNA II under these conditions sediments at 16S (9). Media DNA
in either circumstance showed some heterogeneity in size, with the base of the alkaline gradient spreading from 4-10S. Using a value of $3.6 \times 10^8$ daltons mol wt for SV40 (10), the average double-stranded mol wt of media DNA can be estimated (11) to be about $4.5-6.2 \times 10^8$ daltons. Cell DNA purified and sheared by sonication also sedimented under alkaline conditions in a peak between 7-8S (Fig. 1b).

The $T_m$ of media DNA determined optically differed significantly from that of cell DNA: media DNA $T_m = 83.3$, cell DNA $T_m = 86.6$ (Fig. 2). From the $T_m$ values can be estimated (12) a 42.2% (G+C) content for cell DNA and 35% (G+C) content for excreted DNA. This base pair ratio for cell DNA agrees well with that obtained for lymphoblast DNA (13). Both media and cell DNAs demonstrated about 27% hyperchromicity, which is that expected for double-stranded molecules with 40% (G+C) (8), but occurred throughout a temperature range for media DNA broader than that for cell DNA (Fig. 2). Similar results were obtained when media and cell DNA were subjected to thermal elution from HAP ($T_m$ [HAP], Fig. 3). Cell DNA eluted within a narrow temperature range, 10-15°C, with a $T_m$ (HAP) of 86-87.5%, while media DNA eluted within a much broader and lower temperature range, with a $T_m$ (HAP) of 79-81°C. In an attempt to determine whether two discrete populations of molecules with different base pair ratios might be present in media DNA, thereby causing the broad melting pattern, media DNA was centrifuged to equilibrium in neutral CsCl, but an accurate buoyant density could not be established. Probably because of
its small size, the DNA formed a broad band centered around a density of 1.700 g/cm³.

Reassociation Kinetics. Cellular DNA was extracted and sheared to a size similar to media DNA. Cₛ,ₜ curves for this DNA were then compared to Cₛ,ₜ curves for DNA excreted from PHA-stimulated cells. The curves plot reassociation of DNA from single-stranded to double-stranded form as a function of the Cₛ,ₜ (7), and curves for ideal second order reactions are drawn to reflect the best least-squares solution to fit the data. Since the Cₛ,ₜ-½ (point at which 50% of the DNA has reassociated) is directly proportional to the genetic complexity of the DNA, a Cₛ,ₜ-½ for media DNA smaller than the similarly determined Cₛ,ₜ-½ for total cellular DNA would imply that these DNA sequences are from a limited region of the lymphocyte genome. Escherichia coli, for example, has a Cₛ,ₜ-½ about 1,000-fold less than that found for the unique sequences of mammalian DNA. This difference presumably reflects their relative genetic complexity (7). The Cₛ,ₜ-½ value for a component within a larger population of DNA defined by its reassociation curve is calculated by multiplying the measured Cₛ,ₜ-½ by the fraction of total DNA actually in the given component. This correction has been applied to derive all of the following Cₛ,ₜ-½ values.

Fig. 4 documents the results of such an experiment. 68% of cell DNA reassociates in a single component representing unique sequences, with a Cₛ,ₜ-½ in 0.6 M Na⁺ of 770 mol × s/liter. This result is quite similar to those obtained for human lymphocyte DNA (13, 14) and calf thymus DNA (7) when corrections are made for different Na⁺ concentrations (8) used in those experiments. Media DNA behaves much differently, the major component reassociating at a lower Cₛ,ₜ-½, 53 mol × s/liter in this experiment. The average Cₛ,ₜ-½ for this component from four different media DNA preparations was 87 mol × s/liter (Table II); the
FIG. 4. Reassociation of cell and media DNAs: Media DNA and cell DNA from culture A were concentrated, denatured, and incubated as described in Materials and Methods. Media DNA: 14.4 μg in 24 μl (○); zero time binding = 6%, $C_{50} = 52.5 \pm 1.7$ mol × s/liter, RMS = 1.94%. Cell DNA: 17.9 μg in 15.5 μl (△) or 56.3 μg in 15.0 μl (●); zero time binding 5%, RMS = 1.63%, $C_{50} = 767 \pm 18$ mol × s/liter.

### Table II

| DNA preparation | $C_{50} \times 1/2$ mol × s/liter | Fraction of total in major component | Fraction un-reassociated |
|-----------------|----------------------------------|-------------------------------------|-------------------------|
| A               | 53                               | 0.64                                | 0.14                    |
| B               | 124                              | 0.58                                | 0.22                    |
| C               | 104                              | 0.58                                | 0.20                    |
| "Initial" D     | 68                               | 0.31                                | 0.50                    |
| "Purified" D    | 66                               | 0.36                                | 0.23                    |

Reassociation analyses of media DNAs recovered from the cultures listed in Table I were performed as outlined in Materials and Methods.

sequence complexity of this DNA therefore represents about 10% of the total genome. In each instance about 20% of the media DNA had reassociated by a $C_{50}$ of 1 mol × s/liter; this fraction is assumed to represent repetitive sequences but was not studied further. A variable proportion of the DNA was unreassociated by the end of the experiment (Table II); this could represent DNA degraded to very small fragments or, as demonstrated below for preparation D, "contaminating" cellular DNA of greater complexity.

More experiments were done with preparations B, C, and D to determine whether the DNA in the major component represented unique or repetitive sequences (7) and whether the same sequences were present in media DNA derived from cultures from different cell donors. The technique used was that of Gelb et al. (15) which permits precise measurements of the number of copies of sequences related to a radioactive probe. Known quantities of test DNA, either unlabeled or labeled with $[^3H]$thymidine, are added to marker DNA, labeled with $[^14C]$thymidine. $C_{50}$ values are calculated from the concentration of only marker DNA. In principle, if the test DNA contains sequences similar to the marker DNA, the effective concentration of marker DNA will be increased and
the marker DNA will be driven to reassociate at \( C_{\alpha t} \) values lower than those determined for marker DNA alone. The quantity of similar sequences present in the test DNA can then be calculated (15).

As demonstrated in Fig. 5 A, media DNAs from cultures B and C were allowed to reassociate in the presence of a 10-fold excess of sheared salmon sperm DNA to control for the change in viscosity caused by an increased DNA concentration. In Fig. 5 B, C was allowed to reassociate (a) in the presence of an equal amount of B plus a 10-fold excess of salmon sperm DNA, and (b) in the presence of a 10-fold excess of unlabeled, sheared DNA purified from uncultured human peripheral blood lymphocytes. The reassociation curve for C from Fig. 5 A is included as a reference in 5 B. The expected decrease in \( C_{\alpha t} \) for C was estimated as follows, assuming that sequences in the major components are unique, and, in the case of media DNAs, identical in different preparations:

\[
\frac{\mu g \text{ test DNA} \times \text{fraction in major component}}{\mu g \text{ marker DNA} \times \text{fraction in major component}} \times \frac{C_{\alpha t} \text{ marker major component}}{C_{\alpha t} \text{ test major component}} + 1 = \text{expected acceleration factor}
\]

The expected acceleration factor expresses the theoretical number of copies of marker DNA being added by the test DNA, plus the number of copies of marker DNA already present, assigned a factor of one. The expected \( C_{\alpha t} \) therefore is calculated by dividing the original marker DNA \( C_{\alpha t} \) by this factor. This estimate should be valid where \( C_{\alpha t} \) (test DNA) \( \geq C_{\alpha t} \) (marker DNA). Table
TABLE III

| Experiment | Marker DNA | Test DNA | $C_{av-1/2}$ marker DNA alone | $C_{av-1/2}$ marker plus test DNA | Expected acceleration factor | Measured acceleration factor |
|------------|------------|----------|------------------------------|----------------------------------|-----------------------------|-----------------------------|
| 1          | C          | B        | 104                          | 72                               | 1.84                        | 1.44                        |
|            | C          | Cell     | 104                          | 40                               | 2.60                        | 2.52                        |
| 2          | "Initial" D| B        | 68                           | 23                               | 2.22                        | 2.89                        |
| 3          | "Purified" D| Cell    | 66                           | 26                               | 2.67                        | 2.54                        |

Reassociation of marker DNAs in the presence of test DNAs was performed to determine the number of sequences shared by media DNA preparations derived from different lymphocyte cultures. The techniques of reassociation analysis and calculations to derive expected acceleration factors are described in Materials and Methods. III tabulates the results. Preparation B DNA accelerated the reassociation of C DNA by a factor that was 78% of that expected, demonstrating that most of the sequences present in C were also present in B. DNA in the major components of both B and C must represent unique rather than reiterated sequences, since the addition of a 10-fold excess of cell DNA accelerated the reassociation of C by a factor expected only if one copy of those sequences were present in the cell genome.

Analysis of preparation D is shown in Fig. 6. This preparation was derived from a culture in which the cell number decreased markedly during the culture period, assuming that $10^6$ cells contain 8 $\mu$g DNA (16). A relatively large amount of DNA was present in the media, and, contrasting to most other cultures, the media DNA had a lower specific activity than that of the cell DNA (Table I). Clearly, there had been a greater than usual amount of cell death and lysis. If all media DNA is derived solely from cell death, one would expect the reassociation pattern of D to be no different than that of B and C. This was not the case. In Fig. 6 A, aliquots of the initial preparation of D were allowed to reassociate (a) in the presence of salmon sperm DNA or (b) in the presence of B DNA plus salmon sperm DNA. As noted in Table II and Table III, experiment 2, 31% reassociated with a $C_{av-1/2}$ of 68 mol $\times$ s/liter; B accelerated the reassociation of this component by a factor 130% of that expected if all the sequences present in this component were present once in B. To further characterize the DNA in this component and the 50% of the DNA that did not reassociate within the $C_{av}$ range measured, the entire remaining amount of D was concentrated, denaturated, incubated to a $C_{av}$ of 350 mol $\times$ s/liter, and fractionated on HAP. Single-stranded DNA, representing the unreassociated fraction in the previous experiment, and double-stranded DNA, containing most of the previously reassociated major component plus all of the reiterated sequences reassociating below a $C_{av}$ of 1 mol $\times$ s/liter, were separately collected, dialyzed, precipitated, and studied again by reassociation (Fig. 6 B). The specific activities of the single- and double-stranded fractions (counts per minute per microgram) were identical. The major component in the double-stranded fraction ("purified" D, Table II and
FIG. 6. Reassociation of D media DNA: (A) Culture D media DNA (○), 7.4 µg, was incubated with 74 µg sheared salmon sperm DNA in 16.4 µl; \(C_{\text{t,1/2}} = 67.7 \pm 1.3 \text{ mol x s/liter, RMS} = 0.80\%\). Culture D media DNA (△), 4.5 µg, was incubated with 2.1 µg B media DNA plus 45 µg sheared salmon sperm DNA in 19.1 µl; \(C_{\text{t,1/2}} = 23.5 \pm 0.3 \text{ mol x s/liter, RMS} = 0.82\%\). (B) D media DNA was fractionated before reanalysis: 65.1 µg were concentrated, denatured, made 0.4 M with respect to NaPO₄ pH 6.8 in a final vol of 31.8 µl and incubated to a \(C_{\text{t}}\) of 350 mol x s/liter. The entire sample was separated into double stranded (DS) and single stranded (SS) fractions on a 2-ml HAP column in 0.12 M PB at 60°C, which were then dialyzed, precipitated, and dissolved in 1 mM EDTA. Total recovery was 64%; SS fraction = 27.3 µg, DS fraction, or "purified" D, = 14.1 µg. (a) SS fraction (△), 15.6 µg, was incubated in 15 µl; \(C_{\text{t,1/2}} = 590 \pm 30 \text{ mol x s/liter, RMS} = 2.64\%\). (b) DS fraction (○), 4.4 µg, was incubated with 44 µg sheared salmon sperm DNA in 15 µl; \(C_{\text{t,1/2}} = 65.5 \pm 2.5 \text{ mol x s/liter, RMS} = 1.86\%\). (c) DS fraction (○), 4.4 µg, was incubated with 46 µg sheared lymphocyte DNA in 15.4 µl; \(C_{\text{t,1/2}} = 25.8 \pm 0.8 \text{ mol x s/liter, RMS} = 2.03\%\).

III) had a \(C_{\text{t,1/2}}\) in remarkable agreement with the previously determined value. Although this solution represents the best least-squares fit to the data, it predicts that 23% of the DNA would remain unreassociated. It must be reconciled with the knowledge that all of the DNA in this fraction was double-stranded at a \(C_{\text{t}}\) of 350 when it was separated on HAP from the single-stranded fraction. The apparent discrepancy is explained by recognizing that part of the "contaminating" cellular DNA unique sequences of high complexity also had reassociated at a \(C_{\text{t}}\) of 350 in the first incubation. In the "purified" fraction their concentration would be much lower; when the \(C_{\text{t}}\) calculated for the total population of DNA reached 200 (the last point on the curve), the actual \(C_{\text{t}}\) for these sequences would be approximately 40 (200 x 0.2), and almost no reassociation would have occurred. This explanation is substantiated by the results obtained when the reassociation of "purified" D was driven by a 10-fold excess of unlabeled cell DNA (Table III). The reassociation of this component accelerated by a factor 95% of that expected if its DNA represented unique sequences, but, in addition, the curve fitting the data predicts that only 8% would remain unreassociated (Fig. 6). The unlabeled cell DNA, roughly speaking, increased the concentration of those sequences present in D onefold, but increased the concentration of the contaminating sequences of high complexity 10-fold and acceler-
ated their reassociation into the $C_{ot}$ range measured by the experiment. In contrast, 57% of the DNA in the single-stranded (SS) fraction reassociated with a $C_{ot^{-1/2}}$ of 593 mol $\times$ s/liter; the remainder of its DNA remained unreassociated within the $C_{ot}$ ranges tested.

**Discussion**

My goal in this study was to determine whether DNA found in the media of stimulated lymphocyte cultures has any characteristics that would identify it as more than just the product of cell lysis. The results presented here allow such a distinction to be made.

Media DNA is composed of double-stranded molecules that have an average $T_m$ lower than cell DNA. The reason for this difference is unclear at present. Media DNA as finally isolated consists of predominantly small molecules averaging 7-8S in size, but no information is available regarding the size or composition of this DNA the moment it leaves the cells. DNase activity is readily detected in serum used to supplement the culture media (unpublished observations), and it is possible that the media DNA analyzed here has been degraded from a larger precursor.

The fundamental distinction made between media DNA and cell DNA is based on the results of detailed reassociation kinetics experiments. Media DNA purified from cultures in which active cell growth occurred (preparations B and C) amounted to 10-15% of the total amount of DNA in the culture. In such preparations, about 60% of the DNA reassociated as a major component with a rate indicating a sequence complexity of about 10% of the total genome, 20% reassociated at very low $C_{ot}$ values and was not studied further, and about 20% did not reassociate within the $C_{ot}$ range measured. The sequences comprising the major component were found to be unique rather than reiterated, and, most importantly, 78% of these sequences in media DNA from donor C were present in media DNA from donor B. 30% of media DNA purified from a culture in which considerable cell lysis occurred (preparation D) was comprised of sequences of similar complexity that were unique and were completely shared by media DNA from donor B. This fraction was readily separated from the 50% of the DNA preparation remaining unreassociated at a $C_{ot^{-1/2}}$ of 350 mol $\times$ s/liter. Most of the latter, unreassociated fraction when restudied was found to reassociate with a $C_{ot^{-1/2}}$ of 590 mol $\times$ s/liter. Presumably this fraction was derived from lysed cells.

There are potential errors in these measurements that must be considered before the data can be accepted. Since only small amounts of media DNA were available but high $C_{ot}$ values were necessary, a technique was developed that used very small incubation and sample volumes. Concentration of a 15-μl incubation sample occurred during the course of an experiment; an analysis of a representative experiment expressed as percent concentration was: at 16 h, 0%; 24 h, 1%; 36 h, 15%; 88 h, 26%; 115 h, 37%; and at 115 h, 64%. Since concentration of both DNA and Na$^+$ would substantially increase the reassociation rate, prolonged incubations were avoided. Experiments were constructed so that at least a $C_{ot}$ of 350 mol $\times$ s/liter for media DNA was achieved by 72 h, and all experiments comparing one DNA preparation to another were run in parallel. Much less concentration occurred when larger volumes (e.g., 24 μl) were used.
A more important, potentially large source of error involved analysis of reassociation curves to derive their $C_{ot-\frac{1}{2}}$ values, since in most instances terminal values were not known. Britten's "Finger" computer program (8) seemed admirably suited to this purpose, since it does not require terminal values for accurate rate analysis. The RMS values for the solutions to the data points in the curves presented here are quite low, and the standard errors for the $C_{ot-\frac{1}{2}}$ values are small (see legends to figures), lending statistical probability that those solutions are real. Reproducibility within a set of experiments was high as judged by the close agreement between the $C_{ot-\frac{1}{2}}$ values for "initial" D and "purified" D and by the agreement between expected and measured acceleration factors when the reassociation of C and of D was driven by cell DNA. However, there is an uncertainty imposed upon the quantity of sequences shared by different preparations of media DNA. This can be illustrated by considering the experiment in which the reassociation of C was driven by B to accelerate by a factor 78% of that expected if B contained all of the sequences in C. Theoretically this would result in a biphasic curve for C, with 78% having one rate constant and 22% having a different one. There are too few data points to permit this distinction to be made; the figure 78% is therefore only a reasonable approximation. Similarly, the precision of these experiments probably would not distinguish two components present in any of the media DNA preparations with rate constants differing by a factor of two.

The limitations of these data still permit the following conclusions. Media DNA is derived from two sources. Cell lysis is responsible for a variable amount, but some DNA deserves to be labeled "excreted"; this DNA is composed of a population of unique sequences of limited complexity which are largely shared in preparations from different donors.

Recently Hoessli and Waksman (3) have demonstrated excretion of DNA from lectin-stimulated mouse spleen lymphocytes. Other authors have reported release of DNA from human peripheral blood lymphocytes (17) and rabbit spleen cultures (18), but these studies involved culture conditions that potentially could cause significant cell death. As demonstrated here, not all DNA found in lymphocyte culture media can be assumed to represent excreted sequences.

Lerner et al. have identified DNA, named cmDNA (19) associated with the cytoplasmic membrane of a long term human diploid lymphoblast cell culture line. This DNA has a $T_m$ and a buoyant density similar to that of nuclear DNA (13), but differs in its reassociation kinetics (13, 20). CmDNA reassociates in two components. One, comprising 70% of cmDNA, reassociates rapidly with a low $C_{ot-\frac{1}{2}}$ value and represents repetitive sequences, while the remainder reassociates with a $C_{ot-\frac{1}{2}}$ (corrected to my standard conditions of 0.6 M Na$^+$) of about 60 mol $\times$ s/liter and was shown to consist of unique sequences (20). Excreted DNA and the unique fraction of cmDNA are similar in those respects.

Although excreted DNA represents only a limited part of the cell genome, its sequence complexity is relatively enormous. It is difficult to reconcile any potential function with this fact. Meinke and Goldstein (20) suggested that the unique fraction of cmDNA might be carried along passively by repetitive

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3 Hoessli, D. C., and B. H. Waksman. Personal communication.
sequences which represent the functional portion of that DNA. This theory seems to be at odds with the knowledge that most animal structural genes from which mRNA is transcribed are unique sequence elements (21). Other theories might be constructed for excreted DNA. For example, it is possible that DNA excretion has a role in the immune response since T lymphocytes after initially recognizing an antigen are known to specifically recruit lymphocytes from other subpopulations to respond to that antigen (22, 23). If an individual T lymphocyte excreted only sequences with information somehow specific for the antigen it was programmed to recognize, that DNA would have a very limited complexity. But since PHA nonspecifically stimulates T lymphocytes, the total stimulated population theoretically includes cells specific for a very large number of antigens and the pooled DNA excreted from these cells then could be very complex. This could be tested by comparing DNA excreted by cultured lymphocytes stimulated with different single antigens. Alternatively, each lymphocyte might make single copies of a large set of genes to amplify unknown functions during the process of blast transformation, then excrete these sequences as it differentiates back to the resting state. Stewart et al. (5) have shown that such morphologic reversion, from large blast forms to small differentiated forms, occurs in PHA-stimulated lymphocyte cultures. Experiments to test these two hypotheses are in progress.

Summary

The DNA released into the culture medium after phytohemagglutinin (PHA) stimulation of human peripheral blood lymphocytes has been purified and characterized. It is double stranded, sediments at 7–8S in alkaline sucrose, and has a \( T_m \) determined optically and by thermal elution from hydroxyapatite that is substantially lower than that found for lymphocyte cell DNA. Media DNA contains a major component reassociating with an average \( C_{ot-\frac{1}{2}} \) of 87 mol x s/liter, compared to a \( C_{ot-\frac{1}{2}} \) of 770 mol x s/liter for the unique fraction of cell DNA as measured by reassociation in 0.6 M \( \mathrm{Na}^+ \). This component of media DNA consists of unique sequence elements which are largely shared in media DNA preparations from cultures derived from different cell donors. The marked difference between media DNA and cell DNA indicates that media DNA is not derived from cell death and lysis, rather that some unique portion of lymphocyte DNA is apparently excreted from the cells during PHA-stimulated growth.

I am indebted to Dr. L. Gelb for the gift of SV40 DNA and for helpful criticism of this manuscript. Dr. R. Wette and Ms. M. McCrate, Division of Biostatistics, provided expert statistical analysis and computer programming. Mrs. Fu-Mei Lo provided invaluable technical assistance. Preliminary work on this project was done at the Naval Blood Research Laboratory, Chelsea, Massachusetts, with the support of Dr. C. R. Valeri, officer in charge, and the U. S. Navy.

Note Added in Proof. Anker et al. have claimed recently that resting lymphocytes spontaneously release DNA (Anker, P., M. Stroun, and P. A. Maurice. 1975. Spontaneous release of DNA by human blood lymphocytes as shown in an in vitro system. Cancer Res. 35:2375.).

Reprints of this paper will not be available.

Received for publication 11 December 1975.
References

1. Schellekens, P. Th. A., and V. P. Eijsvoogel. 1968. Lymphocyte transformation in vitro. I. Tissue culture conditions and quantitative measurements. Clin. Exp. Immunol. 3:571.

2. Marshall, W. H., F. T. Valentine, and H. S. Lawrence. 1969. Cellular immunity in vitro. Clonal proliferation of antigen-stimulated lymphocytes. J. Exp. Med. 130:327.

3. Rogers, J. C., D. Boldt, S. Kornfeld, Sr., A. Skinner, and C. R. Valeri. 1972. Excretion of deoxyribonucleic acid by lymphocytes stimulated with phytohemagglutinin or antigen. Proc. Natl. Acad. Sci. U.S.A. 69:1685.

4. Weber, T., C. T. Nordman, and R. Gräsbeck. 1967. Separation of lymphocyte-stimulating and agglutinating activities in phytohemagglutinin (PHA) from Phaseolus vulgaris. Scand. J. Haematol. 4:77.

5. Stewart, C. C., C. F. Cramer, and P. G. Steward. 1975. The response of human peripheral blood lymphocytes to phytohemagglutinin: determination of cell numbers. Cell. Immunol. 16:237.

6. Noll, H. 1969. Polyosomes: analysis of structure and function. In Techniques in Protein Biosynthesis. P. N. Campbell and J. R. Sargent, editors. Academic Press, Inc., New York. 2:101.

7. Britten, R. J., and D. E. Kohne. 1968. Repeated sequences in DNA. Science (Wash. D. C.). 161:529.

8. Britten, R. J., D. E. Graham, and B. R. Neufeld. 1974. Analysis of repeating DNA sequences by reassociation. In Methods in Enzymology. L. Grossman and K. Moldave, editors. Academic Press, Inc., New York. 24:363.

9. Fareed, G. C., E. D. Sebring, and N. P. Salzman. 1972. Cleavage of replicative intermediates of Simian virus 40 deoxyribonucleic acid by the restriction endonuclease of Escherichia coli B. J. Biol. Chem. 247:5872.

10. Tai, H. T., C. A. Smith, P. A. Sharp, and J. Vinograd. 1972. Sequence heterogeneity in closed simian virus 40 deoxyribonucleic acid. J. Virol. 9:317.

11. Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. 11:373.

12. Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5:109.

13. Meinke, W., M. R. Hall, D. A. Goldstein, D. E. Kohne, and R. A. Lerner. 1973. Physical properties of cytoplasmic membrane-associated DNA. J. Mol. Biol. 78:43.

14. Saunders, G. F., S. Shirakawa, P. P. Saunders, F. E. Arrighi, and T. C. Hsu. 1972. Populations of repeated DNA sequences in the human genome. J. Mol. Biol. 63:323.

15. Gelb, L. D., D. E. Kohne, and M. A. Martin. 1971. Quantitation of Simian virus 40 sequences in African green monkey, mouse, and virus-transformed cell genomes. J. Mol. Biol. 57:129.

16. Tedesco, T. A., and W. J. Mellman. 1966. Deoxyribonucleic acid assay as a measure of cell number in preparations from monolayer cell cultures and blood leucocytes. Exp. Cell Res. 45:230.

17. Sarma, D. S. R., and J. Zubroff. 1973. Synthesis and fragmentation of DNA in phytohaemagglutinin-stimulated human peripheral blood lymphocytes. Immunol. Commun. 2:277.

18. Olsen, I., and G. Harris. 1974. Uptake and release of DNA by lymphoid tissue and cells. Immunology. 27:973.

19. Lerner, R. A., W. Meinke, and D. A. Goldstein. 1971. Membrane-associated DNA in the cytoplasm of diploid human lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 68:1212.

20. Meinke, W., and D. A. Goldstein. 1974. Reassociation and dissociation of cytoplasmic membrane-associated DNA. J. Mol. Biol. 86:757.
21. Davidson, E. H., B. R. Hough, W. H. Klein, and R. J. Britten. 1975. Structural genes adjacent to interspersed repetitive DNA sequences. Cell. 4:217.

22. Feldmann, M. 1972. Cell interactions in the immune response in vitro. V. Specific collaboration via complexes of antigen and thymus-derived cell immunoglobulin. J. Exp. Med. 136:737.

23. Taussig, M. J. 1974. T cell factor which can replace T cells in vivo. Nature (Lond.). 248:234.