Replication of Deoxyribonucleic Acid in Lymphoid Cells. An Unusual Effect of Bromodeoxyuridine*

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ABSTRACT: Lymphoid cells which are stimulated to proliferate by antigens or mitogens in the presence of bromodeoxyuridine incorporate DNA precursors into two distinguishable populations of partially substituted DNA molecules which band at densities of 1.710 g/ml and 1.682 g/ml, respectively. The latter fraction, which is distinctly lighter in density than native thymine-containing lymphoid cell DNA, sediments rapidly, as compared with native unsubstituted DNA, in linear sucrose gradients. Both of these unusual properties may be attributable to the presence of lipid in the 1.682 g/ml fraction. This fraction is not observed if bromodeoxyuridine is omitted from the medium.

Reexposure of lymphoid cells to the same antigen used for priming the animal from which they were derived results in a large increase in the amount of bromodeoxyuridine incorporated into the 1.682 g/ml fraction.

Dutton and Eady (1964) have observed that the uptake of [^{14}C]thymidine by lymphoid cell suspension derived from rabbits previously immunized with serum protein antigens is stimulated severalfold by reexposure *in vitro* to the same antigen to which they had been primed. The response is characterized by a lag period for 24 hr following reexposure to antigen. DNA synthesis, as reflected by the ability of the lymphoid cell suspensions to incorporate labeled thymidine then rises to a maximal response on the fourth day. The response appears to be antigen specific, is dependent on antigen concentration, and reflects an increased rate of DNA synthesis and cell division. This system provides a ready means to study the initiation of DNA synthesis without the necessity to synchronize cell populations.

In this report, we present the results of studies on patterns of DNA replication in lymphoid cells using cells derived from guinea pigs previously immunized with selected antigens. Under conditions in which thymidylate synthesis is not limited, the incorporation of [^{3}H]bromodeoxyuridine ([^{3}H]BUdR) is observed into two species of partially substituted DNA which have banding densities close to that of unsubstituted DNA. One of these species has a density of 1.682 g/ml which may be attributable to its relatively high content of lipid. The appearance of this fraction can be induced by antigenic restimulation of primed lymphoid cells, or by the initial exposure of these cells to phytohemagglutinin.

Materials and Methods

Hartley guinea pigs were immunized with selected antigens (ovalbumin, bovine γ-globulin, or hemocyanin), emulsified in complete Freund's adjuvant H-37 Ra (Difco). Emulsion (0.1 ml) consisting of equal volumes of adjuvant and a 5.0 mg/ml solution of antigen was injected into each footpad, thus providing a total dose of 1 mg of antigen per guinea pig.

Two weeks following the primary immunization, the axillary, inguinal, and popliteal lymph nodes were removed under sterile conditions and rinsed once in 10 ml of Eagle's Minimum Essential Medium (MEM) (Microbiological Associates, Bethesda, Md.), containing 100 units/ml of penicillin and 3% isologous guinea pig serum. Nodes from three animals were pooled, and excess fat and connective tissue were removed. The cell suspensions were prepared by teasing the lymph nodes into MEM containing 15% guinea pig serum (GPS). Large tissue fragments were allowed to settle in centrifuge tubes for 10 min, and the supernate was centrifuged at 800 rpm for 10 min. The cells were resuspended in MEM containing 15% GPS and penicillin (100 U/ml) and adjusted to a concentration of 3×10^7 cells/ml.

The standard culture medium employed in these studies contained Eagle's MEM with glutamine, penicillin 100 units/ml, 15% guinea pig serum, cold bromodeoxyuridine (5-Br-2′-deoxyuridine, 0.3 μg/ml), and 1.7 μCi of [^{3}H]BUdR (Schwarz, 12.3 Ci/mm). Antigen, if employed, was supplied at a concentration of 67 μg/ml. Culture medium (1 ml) and the cell suspension (0.5 ml) were added to 100 × 10 mm glass, screw-capped culture tubes and incubated without shaking at 37° in an atmosphere of 5% CO_2–95% air for 20–24 hr. In general, experiments were carried out in sets of 10 tubes containing 10^7 cells/tube.

Preparation of DNA. After incubation, the cells were pelleted by centrifugation and washed twice with normal saline. DNA was then extracted from 1.5×10^8 lymphoid cells. The cells were resuspended in 0.9 ml of 6% paraminosalicylic acid (PAS), frozen, and thawed twice in a Dry Ice–ethylene glycol bath, and homogenized in a Ten-Broeck ground-glass homogenizer with an equal volume of phenol containing 0.1% hydroxyquinoline until the white, stringy nucleoprotein and lipoprotein complexes were dispersed. The solution was shaken for 10 min on a wrist-action shaker and centrifuged to separate phases. The aqueous phase was removed, and the

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interphase was reextracted with PAS. DNA was precipitated with two volumes of cold ethanol, spooled out, and redissolved in 1/10 × SSC (0.015 M NaCl–0.0015 M Na citrate). The DNA was incubated with 50 μg/ml of boiled RNase A (Sigma Chemical Co.) at 37° for 1 hr and then with 100 μg/ml of self-digested pronase (Calbiochem) at 37° for 2 hr. After an additional phenol extraction, the aqueous phase was removed and dialyzed *vs.* 1/10 × SSC overnight. This method yielded a DNA preparation having a sedimentation coefficient ($s_{20,w}$) of 17.4–18.5 S. According to the data of Eigner and Doty (1965), these values correspond to a mean molecular weight of about 5×10^6 daltons.

Sedimentation Velocity. Sedimentation velocity coefficients were determined by analytical ultracentrifugation in SSC at 20° and 29,500 rpm. A plot of the midpoint of the absorption boundary *vs.* time was linear. Several measurements were performed at different concentrations of DNA, and the results extrapolated to zero concentration.

CsCl Density Gradient Ultracentrifugation. The DNAs were prepared for cesium chloride density gradient ultracentrifugation by adding 5.33 g of cesium chloride (Garrard-Schlesinger Co. or Harshaw Chemical Co.) to 4.15 ml of 1/10 × SSC containing 100–250 μg of DNA. The refractive index of the final solution was 1.4000. The solution was centrifuged in a No. 65 Spinco fixed-angle rotor at 36,000 rpm for 44 hr. After centrifugation, 12-drop fractions were collected through a No. 23 needle, and following determination of the refractive index of selected fractions, 0.3 ml of water was added to each fraction and the optical density at 260 mμ was determined. Each fraction was then pipetted with a 50-μl Drummond Microcap onto glass-fiber filters (Reeve Angel, 934AH), which were thoroughly dried prior to counting in toluene–1,4 - bis[2 - (5 - phenyloxazolyl)]benzene–2,5 - diphenyloxazole scintillation fluid using a Packard EX-314 scintillation counter at an efficiency of 27% for tritium. The density of each fraction was computed from the refractive index according to the empirical formula given by Ifft *et al.* (1961).

Sucrose Gradient Ultracentrifugation. Linear gradients (5–20%) of sucrose in 0.05 M phosphate buffer (pH 7.4) were constructed over a 0.1-ml shelf of 60% sucrose in a nitrocellulose tube. Not more than 50 μg of DNA was applied in 0.2 ml of buffer (or less) to the top of the gradient. In selected samples, 0.1 μg (in 10 μl) of a nonsheared preparation of ^{14}C-labeled bacteriophage T2 DNA (10,000 cpm/μg) was included. The gradients were centrifuged in an SW 50 rotor at 36,000 rpm for 2.5 hr at 10°.

Denaturation of DNA. Samples of DNA were denatured either by heating to 95° for 10 min in 0.1 × SSC followed by rapid immersion in an ice bath or by addition of NaOH to a final concentration of 0.15 M. Alkaline cesium chloride gradients were constructed by adding solid cesium chloride to a solution of 0.15 M NaOH followed by addition of the previously denatured DNA sample to be studied. The refractive index of the solution was adjusted to 1.4050 at 25°, prior to centrifugation.

Treatment of DNA with Phospholipase A. Phospholipase A (200 U/mg) was obtained from Boehringer-Mannheim Corp. Lymphoid cell DNA (100 μg) was treated with 25 μg of this enzyme in 2.0 ml of a solution containing the following components per ml: 6.5 mg of Tris, 29 mg of glycine, 25 μg of calcium chloride, and 17 μg of $MgCl_2 \cdot 6H_2O$ for 1 hr at 37°. The pH of the incubation mixture was 8.2. The DNA sample was then subjected to density gradient ultracentrifugation in cesium chloride solution or placed on sucrose gradients.

The phospholipase enzyme preparation was assayed for contaminating DNase activity by incubation of 10–100 μg of the enzyme with 50 μg of a tritium-labeled *B. subtilis* DNA (specific activity = 37,500 cpm/μg) in 0.2 ml of 0.01 M Tris–0.005 M $MgCl_2$ at 37° for 2–24 hr. Samples of labeled *Bacillus subtilis* DNA were incubated under identical conditions, but without added enzyme, as controls. At the end of the incubation period, trichloroacetic acid was added to a final concentration of 5% and the reaction mixture was allowed to stand 1 hr at 4° prior to filtration on Millipore filters. No radioactivity, above the control values, was released into an acid-soluble form by this procedure. By assay of purified DNase (Worthington) 1 mμg of DNase converted 20% of the radioactivity of *B. subtilis* DNA into an acid-soluble form. Thus, the phorpholipase preparation, employed in these studies contained less than 1 part in 25,000 of DNase.

Results

If cultures of ovalbumin-primed cells were reexposed *in vitro* to ovalbumin in the presence of [^3H]bromodeoxyuridine ([^3H]BUdR), the DNA extracted from these cells exhibited a tritium-labeled fraction of light buoyant density in cesium chloride solution. This is shown in Figure 1a, where it is clear that there is, in addition to a tritium-labeled DNA fraction banding at a density of 1.710 g/ml, another labeled band with a mean effective buoyant density of 1.682 g/ml. The measurements of Chun and Littlefield (1963) indicate that the density of a fully substituted hybrid DNA molecule from a mammalian cell line is 1.753 g/ml. Thus, the observed banding density of 1.710 g/ml for the heavier fraction suggests that this species is hybrid DNA in which, under the conditions employed in this study where thymidylate synthesis is not suppressed, the newly synthesized DNA chain exhibits 18–23% substitution of BUdR for thymine. The light-density fraction is indeed unexpected and the balance of this report is devoted to a description of its properties and possible origins.

If ovalbumin-primed cells are similarly incubated in a medium containing no antigen, this light-density DNA fraction was not observed (Figure 1b). Stimulation of ovalbumin-primed cells with the noncrossreactive antigen, bovine γ-globulin, led to the incorporation of only small amounts of the density label into this light DNA peak (Figure 1c). Under conditions of restimulation with the homologous antigen, the amount of tritium label in the DNA of both peaks is increased over that incorporated into the DNA of cells which have been reexposed to an unrelated antigen or to no antigen at all. It is of interest to note that the major fraction of the tritium label incorporated over the base-line level during the first 20 hr following antigenic stimulation *in vitro*, is due to label which appears in the light-density fraction. The appearance of the light-density fraction appears to be related to the degree of antigenic stimulation and presumably reflects the number of lymphoid cells which are undergoing division. The light-density fraction was also observed in the DNA extracted from cells which had been stimulated *in vivo* with hemocyanin 1 week prior to the experiment (Figure 2a). Reexposure of cells primed with hemocyanin to this antigen also resulted in a substantial degree of amplification of the light-density fraction (Figure 2b).

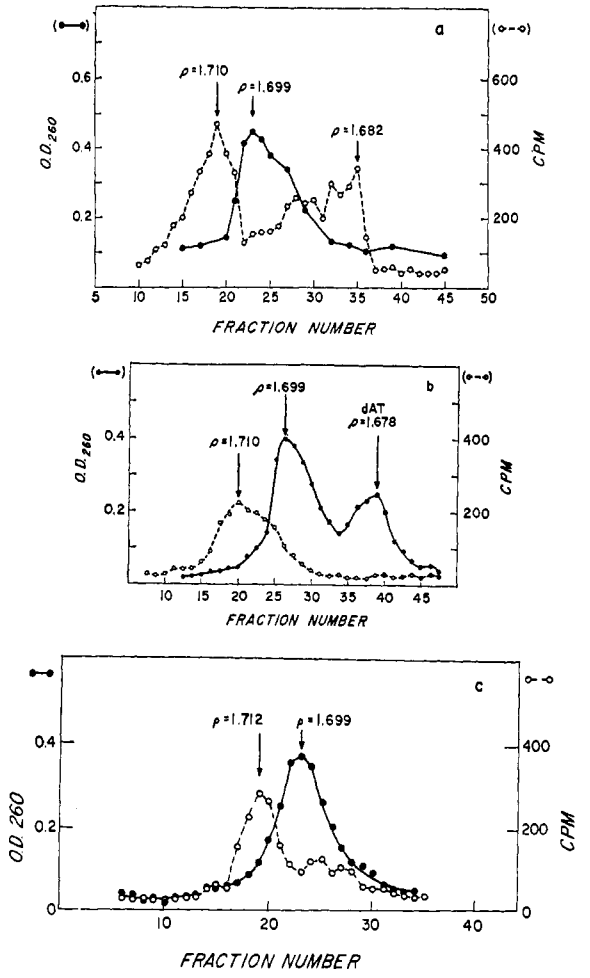

FIGURE 1: Cesium chloride density gradients of DNA derived from cells primed with ovalbumin and reexposed *in vitro* for 20 hr to ovalbumin, bovine γ-globulin, or no antigen in the presence of [^3H]BUdR. Lymph node cells derived from guinea pigs primed with ovalbumin were incubated *in vitro* with ovalbumin (a), no antigen (b), or to bovine γ-globulin (c) in the presence of [^3H]BUdR. The DNA was extracted and banded in a cesium chloride density gradient. The profile of optical density and tritium label are illustrated for each case. A marker of d(A-T) copolymer was included in the preparation of DNA shown in Figure 1b.

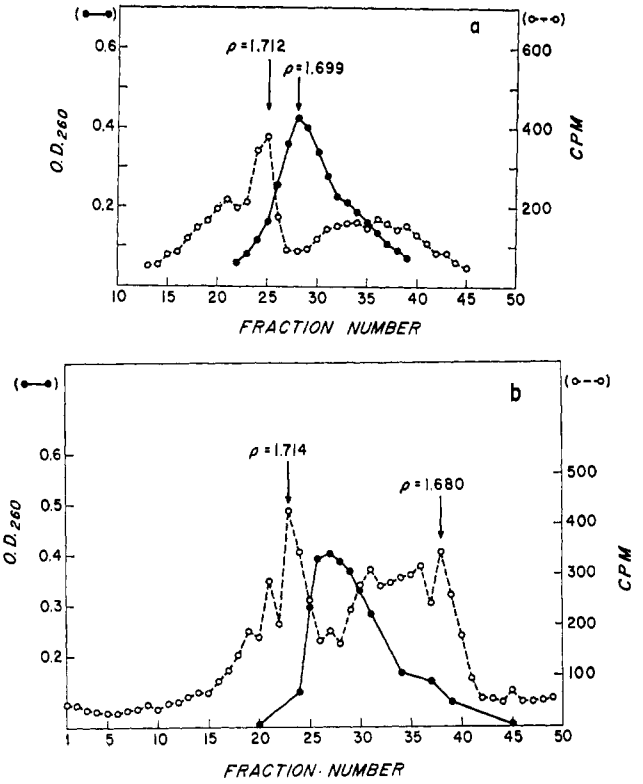

FIGURE 2: Cesium chloride density gradients of DNA derived from cells primed with hemocyanin and exposed *in vitro* to [^3H]BUdR in the presence or absence of hemocyanin. Lymph node cells derived from guinea pigs primed with hemocyanin were exposed to [^3H]-BUdR *in vitro*. DNA (120 μg) from each of these sets of cells was banded in cesium chloride. The profile for the DNA from cells which were not rechallenged is shown in Figure 2a; the profile for the DNA from the restimulated cells is shown in Figure 2b.

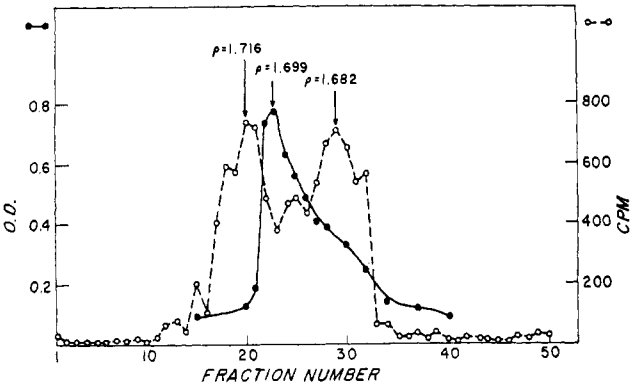

FIGURE 3: Density banding profile of DNA from ovalbumin-primed lymphoid cells exposed *in vitro* to phytohemagglutinin. DNA (100 μg) obtained from a culture of lymphoid cells previously primed with ovalbumin were exposed *in vitro* to phytohemagglutinin (2 μg/ml) in the presence of [^3H]BUdR. The DNA obtained from these cells was centrifuged to equilibrium in cesium chloride solution.

Similar results were observed when guinea pig lymph node cells from primed or unprimed animals were exposed to the mitogen, phytohemagglutinin, *in vitro* for 24 hr in the presence of [^3H]BUdR. Incorporation of the density label into the light-density DNA species was observed to be comparable in all cultures studied regardless of the previous immunization of the donor animal. A representative example is illustrated in Figure 3.

Our attention was then directed to a more detailed characterization of this unusual light-density DNA fraction. A primary concern was to establish that the light-density band did, in fact, represent a unique fraction of DNA and that its appearance in the cesium chloride gradient under these conditions was not a reflection of a technical artifact. Accordingly, the fractions of the CsCl gradient which contained the light-density species and part of the native DNA peak were recentrifuged in a new cesium chloride gradient. The results are shown in Figure 4. The major portion of the light-density fraction rebands to a density of 1.682 g/ml, but there is some contamination with material from the 1.710 g/ml, hybrid peak. It was also noted that the presence of the light-density peak in a given sample of lymphoid cell DNA could also be

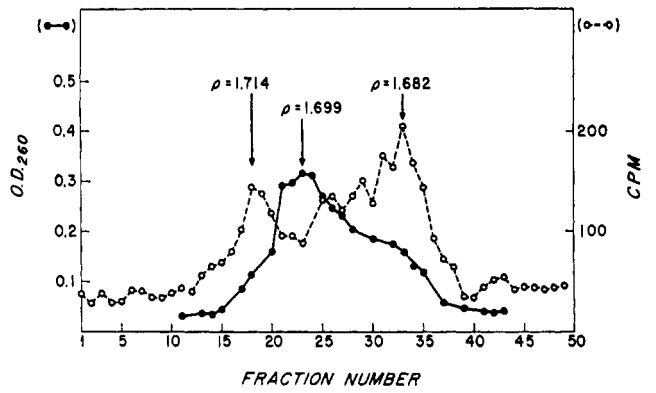

FIGURE 4: Rebanding profile of the light-density DNA fraction. DNA (100 μg) prepared from lymph node cells which had been secondarily stimulated with ovalbumin was subjected to ultracentrifugation in a cesium chloride density gradient constructed so that the fraction at the midpoint had a density of 1.699 g/ml. The fractions on the light side of the native DNA marker and a portion of the marker were dropped directly into another tube containing cesium chloride and the density of that solution adjusted to 1.699 g/ml. After recentrifugation, the optical density and radioactivity of each fraction were determined and are illustrated in this figure.

detected in comparable amounts and at the same density in an SW 50 swinging-bucket rotor. Moreover, the appearance of the light-density band was not influenced by the method of fractionation employed, since collection of fractions by upward displacement of the gradient with mercury yielded the same profile as collection of the gradient by gravity from the bottom of the tube.

The appearance of the 1.682 g/ml light-density DNA species was dependent on the continued presence, at 20 hr after addition of antigen, of bromodeoxyuridine in the culture medium. If cells from ovalbumin primed guinea pigs were reexposed to ovalbumin in a medium containing [^3H]-thymidine but devoid of bromodeoxyuridine the DNA recovered from such an experiment gave the profile shown in Figure 5a. In this case, all of the tritium label appeared in and coincided with the native DNA peak, and no light-density DNA species were observed. However, if cold bromodeoxyuridine was added to a final concentration of 0.5 μg/ml and [^3H]thymidine was again employed as label, a bimodal distribution of ^{3}H label was observed (Figure 6).

When cells were incubated with [^3H]thymidine for relatively brief periods (60–120 min), a fraction of radioactivity was recovered at the top of the gradient as shown in Figure 5b,c. It is of interest to note that the amount of label in this fraction appeared to decline with time. At 20 hr (Figure 5a) no detectable amount of tritium label was seen at the top of the gradient, in the sample of DNA assayed. Friedman and Mueller (1969) observed similar results in synchronized Hela cells pulsed with labeled thymidine.

Denaturation Studies. After heat denaturation, the 1.710 g/ml hybrid peak exhibited a density of 1.734 g/ml in neutral cesium chloride solution. The banding density of this species in alkaline cesium chloride solution was 1.770 g/ml (Figure 7a). These values are consistent with 18–23% replacement of BUdR for thymine in the 1.710 g/ml peak. The light-density DNA fraction, when subjected to denaturation and banding in alkaline cesium chloride solution, also exhibited a banding

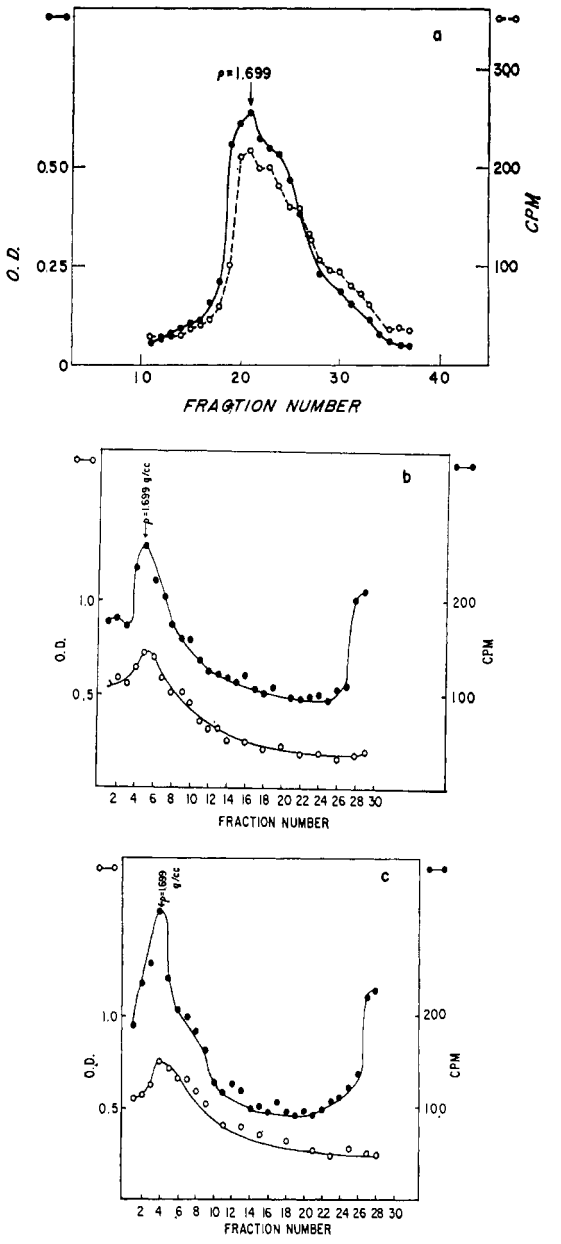

FIGURE 5: (a) Density gradient of DNA derived from cells primed with ovalbumin and reexposed to albumin *in vitro* for 21 hr in the presence of [^3H]thymidine. Lymph node cells derived from guinea pigs primed with ovalbumin were exposed to this antigen in the presence of 2.5 μCi/ml of [^3H]thymidine for 21 hr. The DNA was extracted and 100 μg was banded in a cesium chloride density gradient. The profile of optical density and tritium label are displayed. Density gradients of DNA derived from cells primed with ovalbumin and reexposed to albumin *in vitro* for 60 and 120 min in the presence of [^3H]thymidine. Lymph node cells derived from guinea pigs primed with ovalbumin were exposed to this antigen in the presence of 2.5 μCi/ml of [^3H]thymidine for 60 (5b) and 120 min (5c). DNAs (180 μg) recovered from these cells were banded in a cesium chloride density gradient.

density of 1.770 g/ml (Figure 7b). Upon heat denaturation, the behavior of this fraction in neutral cesium chloride was indistinguishable from that of the 1.710 g/ml species.

These observations indicate that the 1.682 fraction of DNA appeared to be convertible into a form indistinguishable from

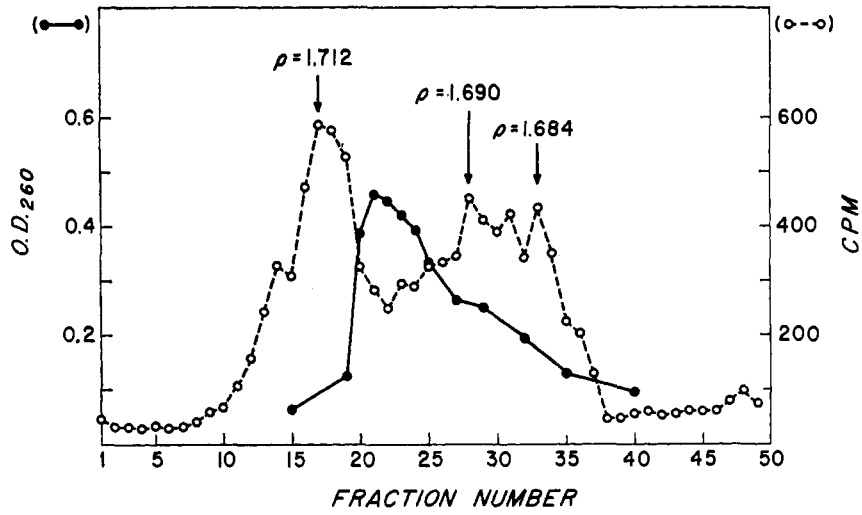

FIGURE 6: Density gradient of DNA derived from guinea pigs primed with ovalbumin and reexposed to this antigen in the presence of [^3H]-thymidine and cold bromodeoxyuridine. Lymph node cells derived from guinea pigs primed with ovalbumin were reexposed to ovalbumin in the presence of 2.5 μCi/ml of methyl[^3H]thymidine and 0.5 μg/ml of cold BUdR. The DNA was extracted and banded in cesium chloride solution. The optical density and tritium level in each fraction are displayed.

the partially substituted 1.710 g/ml hybrid fraction by alkaline denaturation. Since the light-density fraction was not affected by pronase, we reasoned that the unique banding character of this species might be due to lipid. Accordingly, samples of DNA obtained from a hemocyanin-primed lymphoid cell culture which had been reexposed to hemocyanin in the presence of [^3H]BUdR were treated with phospholipase A (shown by assay to be free of contaminating DNase). The result of such treatment is shown in Figure 8. Clearly, the light-density fraction has been abolished by phospholipase and most of the label in that fraction is now seen as part of the total label in the partially substituted 1.710 g/ml fraction. Thus, the unique banding density of the light-density species appears to be attributable to the presence of lipid in this fraction.

Behavior of the Light-Density DNA Fraction on Sucrose Gradients. The behavior of a DNA preparation similar to that shown in Figure 6, which contained the 1.682 g/ml light-

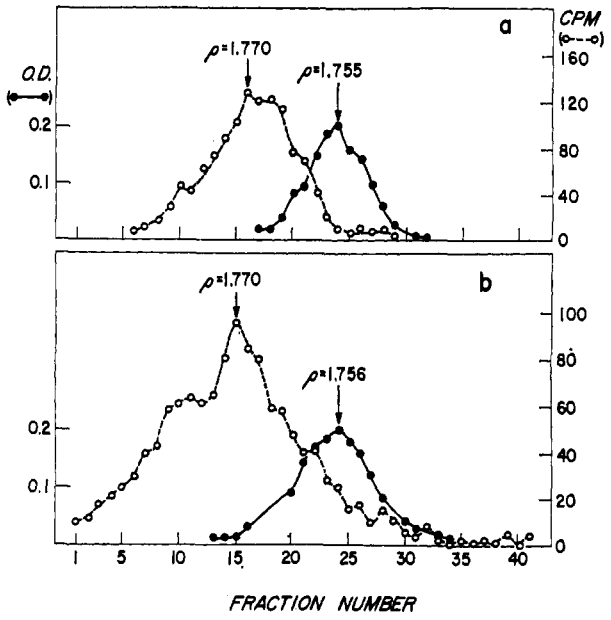

FIGURE 7: Rebanding of lymphoid cell DNA species in alkaline cesium chloride solution. The DNA (100 μg) shown in Figure 2b was centrifuged to equilibrium in a cesium chloride density gradient. The labeled bands on both sides of the native DNA band was recovered, adjusted to 0.1 M in NaOH, and recentrifuged in an alkaline cesium chloride gradient. The profile of the labeled band obtained from the heavy side of the native DNA band is shown in Figure 7a; that of the labeled band obtained from the light side of the native DNA is shown in Figure 7b.

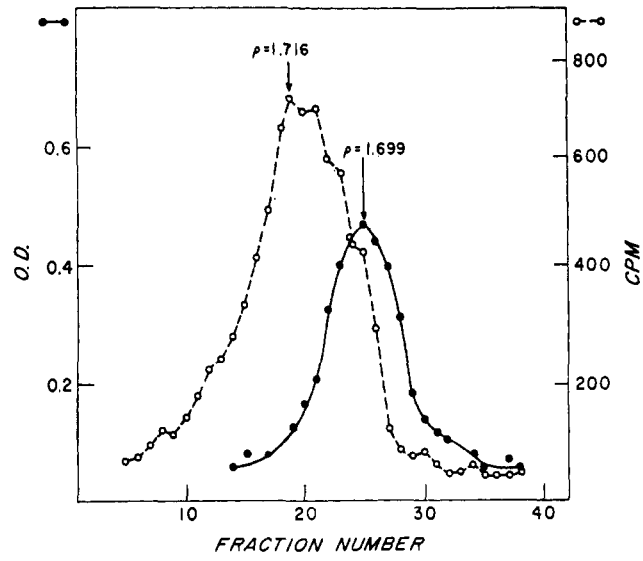

FIGURE 8: Effect of phospholipase A on the bromodeoxyuridine-containing fractions of lymphoid DNA. The DNA (100 μg) shown in Figure 2b was incubated with 25 μg of phospholipase A as described in Methods. The DNA was then centrifuged to equilibrium in a cesium chloride gradient. The profile of the phospholipase-treated DNA is displayed.

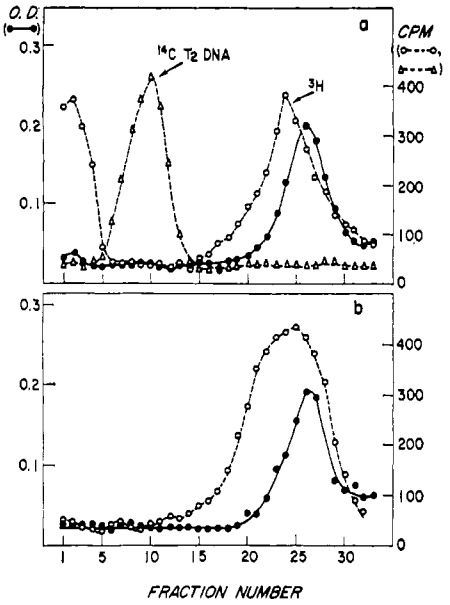

FIGURE 9: Sucrose gradient analysis of DNA derived from guinea pigs primed with ovalbumin and reexposed to this antigen in the presence of [^{3}H]thymidine and cold bromodeoxyuridine. (a) The DNA (33 μg) characterized by the CsCl gradient profile displayed in Figure 6 was centrifuged through a linear (5–20%) sucrose gradient at 35,000 rpm for 2.5 hr at 10°. Drop fractions were collected from the bottom of the tube and the radioactivity and optical density of each fraction was measured. A standard of unsheared [^{14}C]-T2 bacteriophage DNA was included for reference. (b) This DNA (100 μg) was preincubated with 25 μg of phospholipase A for 2 hr at 37° prior to centrifugation of an aliquot of 33 μg over a sucrose gradient.

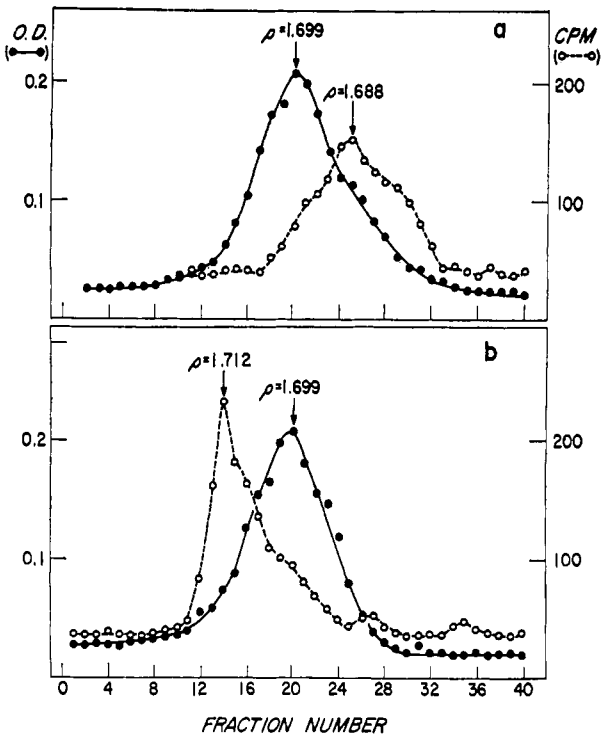

FIGURE 10: Cesium chloride density gradients of DNA fractions derived from sucrose gradient sedimentation of DNA from antigen-restimulated lymphoid cells. The DNA (200 μg) characterized by the profile shown in Figure 6 was divided into three equal parts and centrifuged through sucrose gradients. The rapidly sedimenting and slowly sedimenting fractions were collected separately, pooled, and centrifuged in separate cesium chloride gradients. (a) Banding profile in CsCl of the rapidly sedimenting component from the sucrose gradient exhibited in Figure 9a. (b) Banding profile in CsCl of the slowly sedimenting component obtained from the sucrose gradient exhibited in Figure 9a.

density DNA fraction, on linear gradients is shown in Figure 9a. It is clear that a significant fraction of radioactivity sediments to the bottom of the tube, while most of the label is observed to sediment slightly faster than the native DNA. Pretreatment of the bulk DNA preparation with phospholipase A abolished the rapidly sedimenting component (Figure 9b). It is of interest to note that the enzyme does not affect the sedimentation behavior of the slower sedimenting labeled fraction or of the native DNA in the sample. This indicates that the effect of phospholipase A on the rapidly sedimenting fraction is not due to contamination of the phospholipase preparation with deoxyribonuclease.

When the rapidly sedimenting component was recovered from the sucrose gradient and banded in a cesium chloride density gradient, the profile shown in Figure 10a was obtained. In this profile, it is noted that the radioactivity is found at a density of 1.688 g/ml, and no radioactivity is seen in the heavier fractions. Similarly, rebanding of the more slowly sedimenting labeled material from the sucrose gradient in cesium chloride solution indicates that this material is entirely derived from the 1.710 g/ml fraction (Figure 10b).

The above results establish the existence of a lipid-containing DNA fraction in cultured lymph node cells which are stimulated to proliferate by antigenic or mitogenic stimuli. The appearance of the light-density, lipid-enriched DNA fraction is not uniquely related to antigenic stimulation, but reflects a general characteristic of dividing lymphoid cell populations.

Discussion

In mammalian cells, it has been suggested that the process of DNA replication is a nonrandom focalized phenomenon at the chromosomal level (Taylor, 1968; Stubblefield and Mueller, 1962). A model of DNA replication in Chinese Hamster cells has been proposed by Huberman and Riggs (1968) based on autoradiographic studies of [^{3}H]thymidine uptake into the chromosomal component of these cells. This model suggests that the long fibers of DNA are made up of many tandemly joined replication sections. The synthesis of DNA begins at the midpoint of large units of replication and proceeds bidirectionally to the junctions of the two adjacent replication units. In analogy with bacteria, Comings (1968) has suggested that the chromosomes are attached at multiple points to the nuclear membrane. Each point of attachment represents the initiation site for DNA synthesis in the replicating unit.

Our observations indicate that in the 24 hr following stimulation of lymphoid cells with antigens or mitogens, two classes of newly replicated DNA may be distinguished in gradients of cesium chloride. The first of these which is seen on the heavy side of the native DNA marker appears to be DNA consisting of one parental strand and one newly replicated strain in which the substitution of BUdR for thymine

is incomplete. The second species is distinguished by an unusually light density (1.682 g/ml) in cesium chloride solution. This property appears to be related to the presence of lipid in this DNA fraction, as the unique banding property of this species was destroyed by incubation with phospholipase A, while it was unaffected by incubation with pronase.

Friedman and Mueller (1969) have observed a lipid-enriched DNA component in synchronized cultures of Hela cells. Centrifugation of newly replicated DNA from these cells revealed that the labeled material separated into two fractions: one which sedimented rapidly to the bottom of the sucrose gradient but floated in cesium chloride solution; and another fraction which sedimented somewhat slower than nonreplicated DNA in a sucrose gradient but had the same density as parental DNA in cesium chloride. The rapidly sedimenting form was converted into a slower sedimenting material with the same density as parental DNA by heat, alkali, or sonication, but was not affected by pronase. Many of these characteristics are shared by the 1.682 g/ml fraction found in our studies. Moreover, when lymphoid cells are cultured with antigen and [^3H]thymidine for periods of 60–120 min, a significant fraction of the label appears to float at the top of the gradient. This very light-density fraction appears to contain less and less of the total thymidine incorporated as the incubation is continued. The relationship of this material to the 1.682 g/ml fraction seen when BUdR has been present for 20 hr is not established. It is possible that the 1.682 g/ml material is the BUdR-containing analog of the replicating DNA fraction which is observed to float in our cesium chloride system. If this is so, our observations suggest that BUdR seems to cause an accumulation of the 1.682 g/ml material possibly by blocking the continuation of the growth of the nascent chain beyond the membrane initiation site. Thus, the 1.682 g/ml fraction may be a relatively short segment of newly replicated DNA attached to a portion of the nuclear membrane. Such a complex could arise by BUdR-induced termination of the nascent DNA chain.

Haut and Taylor (1967) have suggested another mechanism. They studied DNA replication in root cells of *Vicia faba* cultured in the absence of inhibitors of thymidylate synthesis. They observed the appearance of labeled BUdR into two fractions which distribute bimodally, in cesium chloride gradients on either side of unsubstituted DNA. These fractions appear similar to ours. As Haut and Taylor suggest, these two density species of DNA which appear only when bromodeoxyuridine is present can be accounted for by assuming insertion of a short primer chain at the origin of each replicating unit and that there are at least two primers in each unit with widely different thymine contents.

While it appears that the incorporation of DNA precursors into a lipid-enriched DNA fraction is not uniquely related to antigenic stimulation or to replication of DNA in lymphoid cells specifically, our results are relevant to a description of the early events following challenge of lymphoid cells with antigen. In this report, we note the amount of the light-density DNA species formed by primed lymphoid cells is greatly increased upon reexposure of these cells to the priming antigen. Indeed, the major proportion of the [^3H]BUdR observed in the bulk DNA from these cells appears in the light-density DNA species. In the studies of Dutton and Parkhouse (1965), it was observed that the maximal incorporation of labeled thymidine into lymphoid cells was observed 2–4 days after reexposure to the priming antigen. In Dutton's studies, the 24-hr period immediately following antigen reexposure was actually one characterized by a decline in thymidine incorporation. Nevertheless, our studies indicate that a lymphoid cell population exhibits relatively specific recognition of a priming antigen in the first 24 hr following reexposure to antigen, as indicated by the striking incorporation of [^3H]-BUdR into the light-density DNA species. While the significance of this species is unclear, the probable location at or near the site of replication suggests that it may play an important role in the early phases of the replication process.

Added in Proof

Recently, Souliel and Panijel (1970) have reported on studies similar to those described in this paper. These authors also observed a light-density BUdR-containing species under conditions of homologous antigen stimulation. In contrast to our studies, such a species was not obtained in their phytohemagglutinin-stimulated cultures. This may reflect differences in the efficacy of the phytohemagglutinin preparations employed in their work and ours. Although Souleil and Panijel made no attempt to characterize the light-density species observed in their system, they have concluded that this species reflects a population of partially substituted DNA fragments which differ from those of hybrid DNA in the degree of replacement of BUdR for thymine. These authors interpret their results as being due to a preferential replication of a large number of genes concerned with the process of antibody formation.

In view of the evidence presented in this paper, the conclusions of Souleil and Panijel appear untenable. All of the evidence presented contradicts the *ad hoc* assumption of these workers that antigen stimulation selectively modifies the normal pattern of DNA replication.

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