SHORT TERM INCUBATION WITH BROMODEOXYURIDINE OF ANTIGEN- OR PHA-STIMULATED CELLS

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1. Introduction

We have reported that DNA extracted from antigen-stimulated cells cultivated in the presence of 5-bromo-2-deoxy-[^{3}H]uridine ([^{3}H]BrdU) bands on CsCl gradients with a particular bimodal distribution [1]. The radioactive density label is incorporated both into hybrid DNA – as can be expected from the semi-conservative pattern of DNA replication – and in a second peak of lesser density. However, after phytohemagglutinin (PHA) stimulation of the cells, all the label is found in a single peak of hybrid density. On the basis of proteolytic or lipolytic digestion, denaturation studies and sedimentation in the presence of a detergent, we have rejected the hypothesis that, in antigen-stimulated cells, a low density substance remains attached to a fraction of hybrid density fragments after DNA extraction [2]. On the other hand, ultrasonic treatment of the DNA, which causes all the radioactive label to band at hybrid position in a single peak, demonstrates that the second labelled peak contains end-to-end junctions of labelled and unlabelled segments. This results in the formation of DNA fragments of a lesser degree of substitution than hybrid DNA.

When cells are grown in a bromodeoxyuridine containing medium, four classes of chromosomal fragments are theoretically expected to have densities intermediate between normal and hybrid DNA, the density label being present in only a part of the fragment: i) fragments containing the transition point (i.e. the position of the growing point at the instant of transfer to the BrdU medium; b) fragments containing the replication point (caught at the instant the culture was terminated); iii) fragments containing junctions of already replicated units with non-replicated ones; iv) fragments involved in a non semi-conservative replication process such as repair replication. Short term incubations with 5-bromo-2-deoxy-[^{3}H]uridine, followed by chase with cold 5-bromo-2-deoxy-uridine, were performed in the hope that the results could shed some light on the significance of the bimodal profile in CsCl gradients of density labelled DNA extracted from antigen-stimulated cells.

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2. Materials and methods

2.1. *Immunization*

Hartley guinea pigs were immunized with a total of 100 μg of Keyhole Limpet Hemocyanin (KLH) (Calbiochem, immunologically pure) in complete Freund's adjuvant.

2.2. *Tissue culture*

A minimal delay of 3 weeks was observed between *in vivo* immunization and preparation of a cell suspension from the lymph nodes, as previously described [1]. The dissociated cells were resuspended in tissue culture medium (minimum Eagle essential medium, GIBCO, supplemented with non-essential amino-acids, 1 mM Na pyruvate, 15% normal guinea pig serum, and containing 100 units/ml penicillin); cultures with $1-1.5 \times 10^7$ cells in 1.5 ml medium were incubated in a 5% CO_2–95% air atmosphere, with 100 μg/ml KLH or 0.01% phytohemagglutinin-P (PHA-P from DIFCO, Michigan).

2.3. *Incubation with the density label*

The cultures were incubated for 28 hr or 48 hr with the antigen or PHA. All the following steps were carried out at 37°. The cells were gently resuspended and 0.15 ml of 5-fluoro-2-deoxyuridine, 5 μg/ml (a gift from Hoffman-LaRoche) was added to each culture to deplete intracellular pools of thymidilate. After 15 min, 0.1 ml of a solution of 5-bromo-2-deoxy-[^{3}H]uridine ([^{3}H]BrdU) 45 μCi/45 μg/ml was added and the incubation in 5% CO_2–95% air carried out for 30 to 90 min. At that time, antigen- or PHA-stimulated cells from 10 culture tubes were pooled, centrifuged and frozen until DNA extraction. Two other sets of ten antigen- or PHA-stimulated cultures were centrifuged at 800 rpm for 8 min. The supernatant was removed and replaced by 1.5 ml of fresh medium containing 3 μg/ml cold BrdU and 2×10^{-6} M 5-fluoro-2-deoxy-uridine. Further incubation was carried out for a length of time equal to that already done in the presence of the radioactive density label. Then, the culture was terminated by centrifugation of the cells, which were also frozen until DNA extraction. In some experiments, antigen- or PHA-stimulated cells were incubated for 30 hr from time zero of the culture (defined by the addition of the mitogen) with 1 μCi/3 μg/ml [^{3}H]BrdU and 2×10^{-6} M 5-fluorodeoxypuridine.

2.4. *DNA extraction and CsCl gradients*

DNA was extracted by a modification of the Kirby method as previously described [1]. The DNA was banded in neutral CsCl gradients using the SW 50.1 swinging bucket rotor, fractionated, and the refractive index, the optical absorbance at 260 nm, and the radioactivity in each fraction estimated as already reported [1].

3. Results

Fig. 1 shows the banding profile in a CsCl gradient of DNA extracted from antigen- (fig. 1a) or PHA- (fig. 1b) stimulated cells, cultivated for 48 hr with [^{3}H]BrdU. As only a fraction of the cells, when stimulated, replicate DNA, the absorbance peak corresponds to the normal, unsubstituted DNA, and bands at a

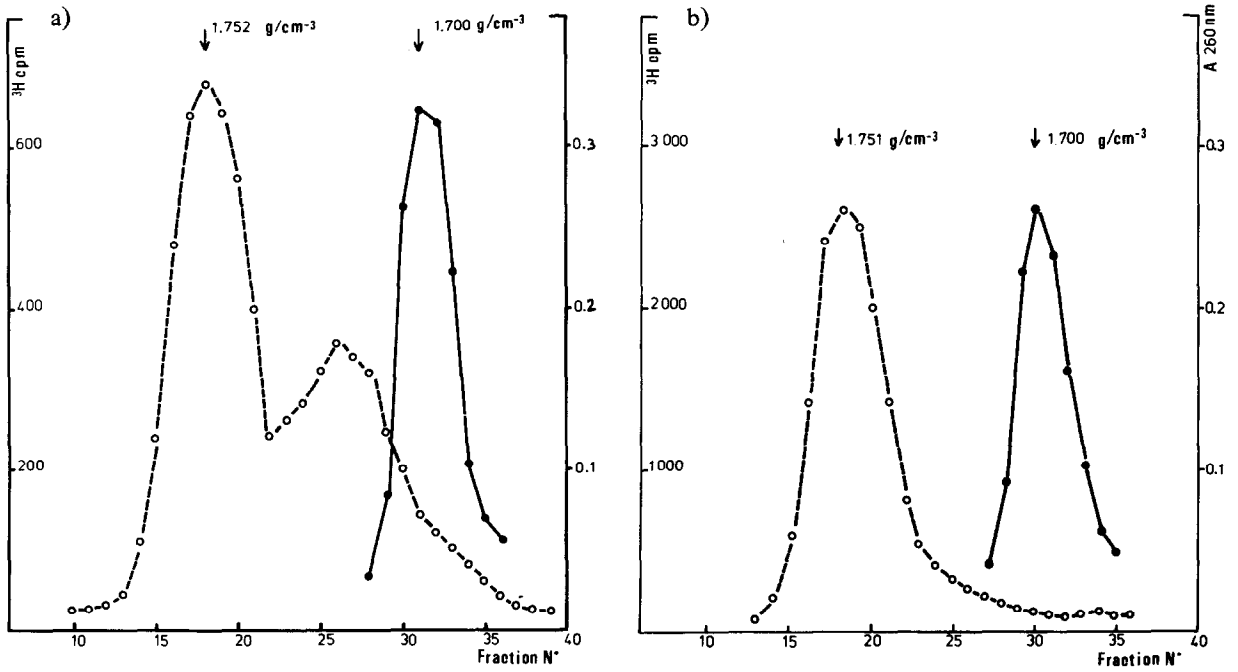

Fig. 1. CsCl gradients of DNA extracted from primed guinea pig lymph node cells after 48 hr of incubation with homologous antigen or PHA-P, [^{3}H]BrdU 1 μCi/3 μg/ml, 2×10^{-6} M 5-fluoro-2-deoxy-uridine. (●—●—●) Optical absorbance at 260 nm; (o—o—o) radioactivity. a) Stimulation with 100 μg/ml Keyhole Limpet Hemocyanin; b) stimulation with 0.01% PHA-P.

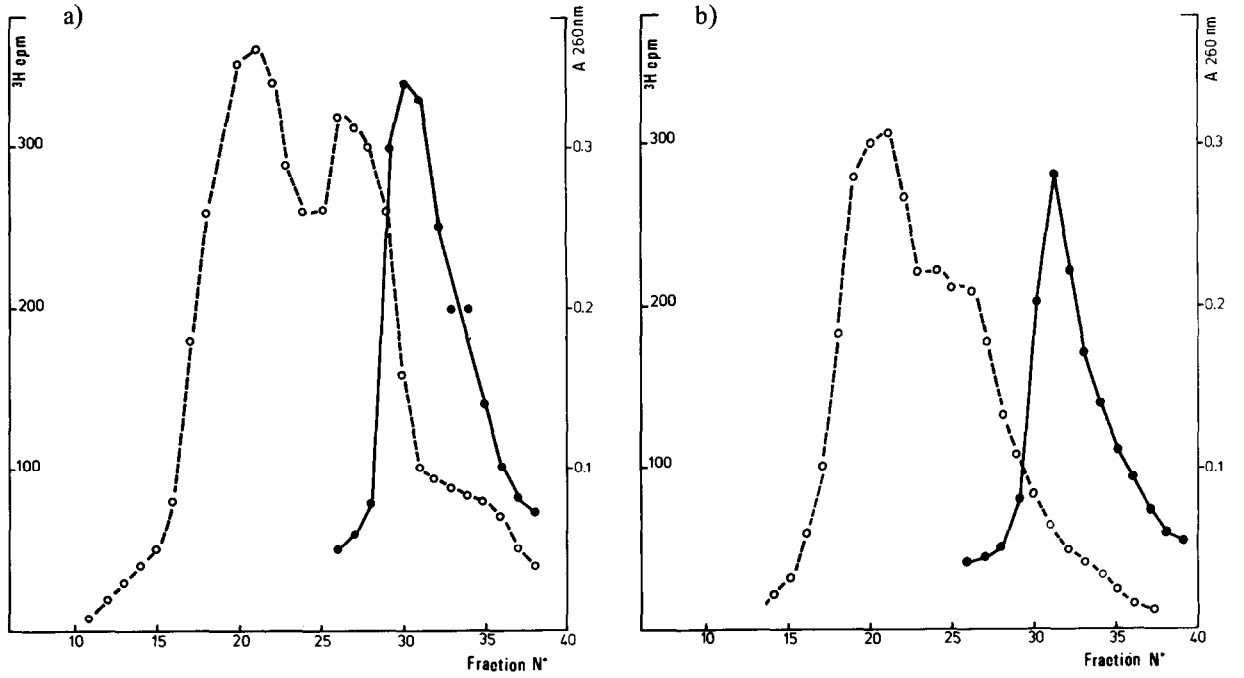

Fig. 2. CsCl gradients of DNA extracted from primed guinea pig lymph node cells, incubated for 28 hr with the antigen or PHA. Then, the tissue culture medium was made up 3 μCi/3 μg/ml [^{3}H]BrdU and 2 $\times 10^{-6}$ M 5-fluorodeoxyuridine, and the incubation carried out for an additional 1.5 hr. a) Stimulation with 100 μg/ml Keyhole Limpet Hemocyanin; b) stimulation with 0.01% PHA-P. Legends as in fig. 1.

density of 1.700 gcm^{-3}. In the conditions reported here, (sufficient supply of an exogenous analog in the presence of 5-fluoro-2-deoxy-uridine as inhibitor of thymidilate endosynthesis) complete substitution of thymine by bromouracil in replicating DNA should take place [3]. Indeed, the heavier peaks of labelled, newly synthesized DNA in fig. 1a and b have a density of 1.752 gcm^{-3} and 1.751 gcm^{-3}, respectively, which indicates an almost complete substitution of thymine by bromouracil. As already described [1], an intermediate density peak is seen when the DNA is extracted from antigen-stimulated cells (fig. 1a), absent in PHA-stimulated cells (fig. 1b).

Fig. 2a and 2b show CsCl gradients of antigen- or PHA-stimulated cells incubated for 1.5 hr with 5-bromo-2-deoxy-[^{3}H]uridine. Unlike the conditions in fig. 1, where the density label is added with the stimulant for DNA synthesis (so that BrdU can be expected to be incorporated from the origin of replicating units) a 28 hr period of incubation with the antigen or PHA alone was used before addition of the density label. By this time, the lag period is over and DNA synthesis occurs in stimulated cells [4]. Therefore, when the cells are transferred into a [^{3}H]BrdU containing medium and then the DNA extracted and banded in a CsCl gradient, an appreciable amount of DNA fragments can be expected to contain transition points (as defined above). Since several separated units have been shown to replicate simultaneously in mammalian cells [5], they should therefore band at a density intermediate between normal and hybrid DNA on a CsCl gradient. Indeed, in addition to the hybrid peak, an intermediate density peak is present both in antigen-stimulated cells (fig. 2a) and in PHA-stimulated cells (fig. 2b). The relative amount of radioactivity incorporated in the intermediate density peak, as compared with the hybrid density peak (which is heavier), is higher in antigen-stimulated cells than in PHA-stimulated cells.

DNA fragments containing the replication point band also at an intermediate density since they are formed by a fully substituted segment joined to an

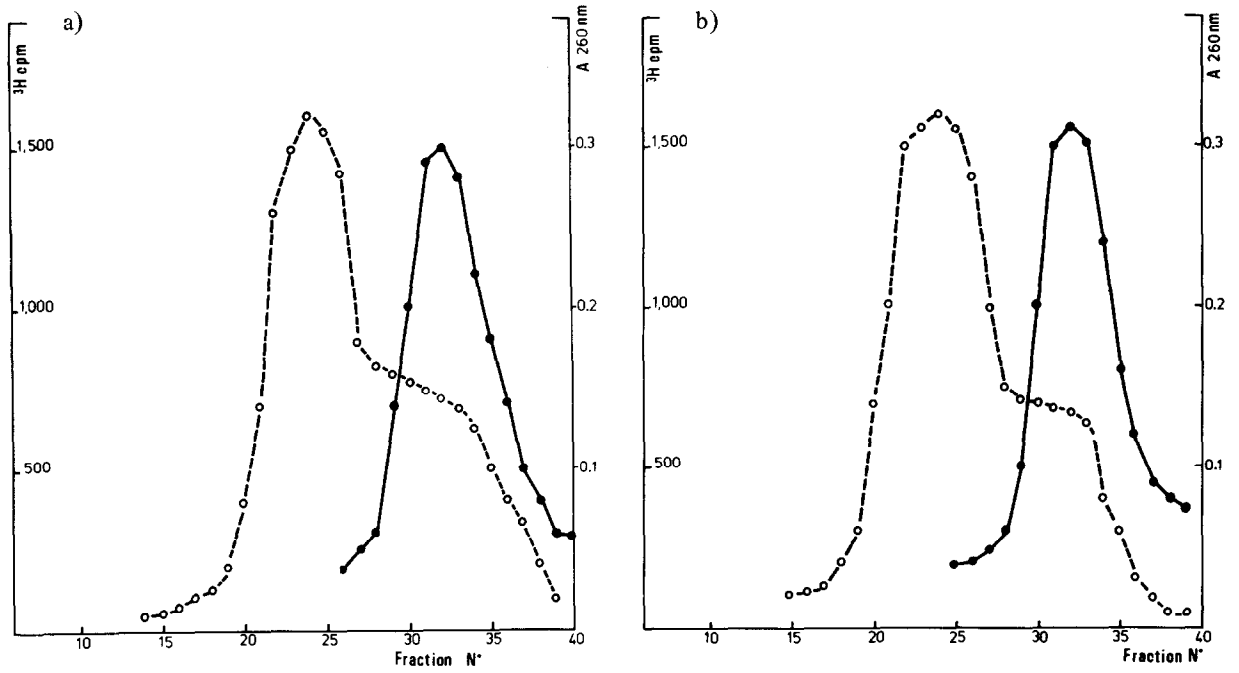

Fig. 3. CsCl gradients of DNA extracted from primed guinea lymph node cells, incubated as in fig. 2. After the radioactive density labelling, the cells were transferred to a medium containing 3 μg/ml cold BrdU, and the incubation carried out for a further 1.5 hr. Fig. 3a and 3b, and legends as in fig. 1.

unsubstituted one [6]. To test whether some of the fragments obtained after short incubations with [^{3}H]BrdU were of this kind, cultures were transferred in cold BrdU for 1.5 hr. In these conditions, as growing points continue to move along the chromosome, cold BrdU is used to build up the chains so that the partially substituted fragments should be chased as complete substituted fragments if containing the replication point. Fig. 3a and 3b show the results of this experiment. The intermediate density peak is still present, but the radioactivity incorporated has diminished, and this phenomenon is more pronounced in antigen-stimulated cells (fig. 3a).

When short incubation and chase experiments are made after 48 hr of incubation with the stimulant, the results are comparable, which can be expected in an asynchronously dividing cell population, where new cells are continuously involved in the process as the incubation is prolonged.

4. Discussion

The results reported in this paper indicate that only a small fraction of the intermediate density fragments contain replication points after an incubation of 1.5 hr. However, this estimation is made difficult by the fact that they are very sensitive to shear [6]. Other intermediate density fragments in a semi-conservative pattern of DNA replication should contain transition points or junctions of already replicated units with non replicated ones. As the rate of chain growth had been estimated from 0.5 to 1.5 μm/min [7] an appreciable amount of the last kind of fragments can be predicted. However, the results reported here do not allow any distinction between them and fragments containing transition points.

The differences found between antigen-stimulated and PHA-stimulated cells suggest tht a larger fraction of the replicons would be operating in antigen-

stimulated cells at any moment, or that replicons are larger in PHA-stimulated cells. The fact that, when BrdU is present from the beginning of the stimulation, a single peak is present in PHA-stimulated cells would favour the second hypothesis.

On an other hand, a repair mechanism would hardly lead to such an important amount of intermediate density fragments in such a short time. The fact that this amount is higher in short incubations than in long incubations shows that the synthesis of new chains is involved.

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