Quantitative Nucleic Acid Changes during Phytohaemagglutinin-Induced Lymphocyte Transformation *in vitro*

DEPENDENCE OF THE RESPONSE ON PHYTOHAEMAGGLUTININ/SERUM RATIO

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1. Pig and human blood lymphocytes have been grown in culture without replenishment of medium, and stimulated to transform by phytohaemagglutinin. Quantitative nucleic acid changes during this process have been used as an index of transformation. 2. On the first day, cells attach to glass; then they detach and continue transforming. 3. The degree of transformation is dependent on the phytohaemagglutinin/serum ratio, and is independent of cell concentration within the range 0.5×10^6 – 2.0×10^6 cells/ml. 4. At low phytohaemagglutinin/serum ratios there is no response; at high phytohaemagglutinin/serum ratios, inhibition appears after the cells have been cultured for a day.

Circulating mammalian lymphocytes are of importance in the primary immune response (McGregor & Gowans, 1963). Under the influence of various stimulants, they may be induced to transform, while being cultured *in vitro*, from small cells containing little cytoplasm into large pyronine-staining 'blast' cells (rich in ribosomes), which divide (Robbins, 1964; Johnson & Roberts, 1964). Similar cytological changes are seen within lymphoid organs during various immune responses (Fagraeus, 1948; Scothorne, 1957; Gowans, 1962; Oort & Turk, 1965).

A high percentage of the lymphocytes present in a culture transform when stimulated by PHA,* which is an extract of the beans of *Phaseolus vulgaris* (Nowell, 1960), and similar responses may be obtained with streptolysin-S (Hirschhorn, Schreibman, Verbo & Gruskin, 1964), a culture filtrate of *Staphylococcus aureus* (Ling, Spicer, James & Williamson, 1965), or anti-lymphocyte antibody (Grasbeck, Nordman & de la Chapelle, 1964). Smaller percentage responses have been observed in the presence of other agents (Sell & Gell, 1965; Bain, Vas & Lowenstein, 1964; Pearmain, Lycette & Fitzgerald, 1963; Johnson & Russell, 1965).

To relate the phenomenon of transformation *in vitro* to immune responses *in vivo*, and to examine the cellular control mechanisms involved, an initial study has been made of the quantitative nucleic acid changes occurring when blood lymphocytes from pigs and humans are stimulated by PHA. The period over which cultures may be left without replacement of medium, and the roles of variables such

as PHA concentration and serum concentration, have been examined. Preliminary accounts of this work have appeared elsewhere (Forsdyke, 1966*a,b,c*, 1967).

MATERIALS AND METHODS

Tissue culture medium '199'. This was obtained from Burroughs Wellcome and Co., London, W. 1, in a form modified from that used by Morgan, Morton & Parker (1950). To the medium were added (final concentrations): NaHCO_3 (13 mm); sodium benzylpenicillin (200 units/ml.); streptomycin sulphate (50 $\mu\text{g./ml.}$).

PHA. A crude red-kidney-bean extract, prepared as described by Hurn (1966), was obtained from Burroughs Wellcome and Co. The bottles contained approx. 50 mg. of powder, which was dissolved in 0.14 M-NaCl (1–5 ml.). Partially purified preparations of PHA used in some experiments were obtained from Difco Laboratories, Detroit, Mich., U.S.A. ('PHA-P', optimum 0.04–0.2 mg./ml. of serum), and Burroughs Wellcome and Co. (optimum approx. 0.04 mg./ml. of serum).

Chemicals. Gelatin, obtained from Harrington Brothers Ltd., London, E.C. 1, was dissolved in 0.14 M-NaCl at 45° to a final concentration of 3% (w/v) and Seitz-filtered. Buffered salt solution, used to wash cells, contained (final concentrations): NaCl (140 mm); KCl (4 mm); CaCl_2 (2 mm); MgCl_2 (1 mm); tris-Cl, pH 7.4 (10 mm). Orcinol reagent consisted of 0.1% (w/v) orcinol and 0.1% (w/v) FeCl_3 in A.R. conc. HCl (sp. gr. 1.18).

Glassware. Cultures of 1 ml. volume were kept in glass round-bottomed centrifuge tubes of 15 ml. total capacity and 1.4 cm. internal diameter. Cultures of 5 ml. volume were kept in glass screw-cap flat-bottomed universal containers of 30 ml. total capacity and 2.4 cm. internal diameter. Flat medicine bottles of various sizes were used for cultures of larger volume.

Lymphocyte cultures. Precautions to prevent contamination by micro-organisms were taken. All the cell counts

* Abbreviation: PHA, phytohaemagglutinin.

refer to values obtained at the initiation of cultures. All centrifugations were at 1500g for 2–3 min., unless otherwise stated. Suspensions of lymphocytes in autologous serum were prepared from freshly defibrinated blood by a gelatin-sedimentation procedure (Coulson & Chalmers, 1964; Thomson, Bull & Robinson, 1966). Pig blood was collected into a Thermo flask containing penicillin and streptomycin in the same final concentrations as in the tissue-culture medium, and was gently defibrinated for 10 min. by rotating a bent glass rod that passed through a hole in the cap of the flask. (This procedure removes platelets and some polymorphonuclear cells, as well as various clotting factors; it is possible that cell destruction and release of unknown factors into the serum may also result.) Then 0.33 vol. of gelatin solution at 37° was added. After 30–60 min., the upper layer above the erythrocyte sediment was siphoned off. Approx. 80% of the nucleated cells present were small or medium lymphocytes; extremes of 50% and 95% were occasionally obtained. Contaminating cells were mainly polymorphonuclear. Other cells observed were large lymphocytes, and larger poorly staining cells that may have been monocytes (Yoffey, Winter, Osmond & Meek, 1965). (Erythrocytes were present in all the cultures; the ratio of erythrocytes to lymphocytes was approx. 10:1.) When necessary, any excess of cells was removed by centrifuging and the serum added back to give a concentration of 3×10^6 cells/ml. Then 2 vol. of culture medium at 37° was added to give a final cell concentration of 10^6 cells/ml. Since the serum had been diluted with 0.33 vol. of gelatin in 0.14M-NaCl during the preparation procedure, the final serum concentration was 25%. Appropriate volumes of cells in suspension were pipetted in wide-bore silicone-treated pipettes. After the addition of PHA [generally 0.6 mg. of crude PHA (Wellcome)/ml. of serum], culture vessels were gassed briefly with an air+CO₂ (19:1) mixture and sealed with silicone-rubber bungs or silicone-rubber-lined screw-caps. A period of 1½–5 hr. usually elapsed between the collection of the blood and the final addition of PHA. Cell suspensions were maintained at 37° except when being pipetted at room temperature. Usually a depth of 1 cm. separated the cell sediment from the gas phase. The cultures were incubated in darkness at 37° without agitation. Since the medium was not replenished, nutrient growth factors were either present initially or were released from degenerating cells. By the second day culture, most of the cells had transformed. In the absence of PHA no such transformation was seen.

At appropriate times, warm cultures were centrifuged and the supernatants carefully poured off. The cell pellets were washed by resuspension in buffered salt solution at 0° to remove components of the medium that interfere with the RNA assay by decreasing the E_{260}/E_{240} ratio. After a second spin the pellets were stored in ethanol at –10°. When cells were spun down, clumps of erythrocytes sedimented faster than clumps of lymphocytes and two layers were visible in the cell pellets. This is in accord with the report that clumps containing both red and white cells are not found when PHA is added to cells in serum (Nordman, de la Chapelle & Grasbeck, 1964). The red layer was no longer visible when the cells had been cultured for 5 days. Centrifugation within a few minutes of the addition of PHA produced a dispersed cell pellet, in contrast with the compact pellet obtained from unstimulated cultures.

Measurement of RNA. The Schmidt & Thannhauser

(1945) procedure, as modified by Scott, Fraccastoro & Taft (1956), was adapted for measuring lymphocyte RNA as recommended by Hutchison & Munro (1961) and Fleck & Begg (1965). Cell pellets containing 1×10^6 – 5×10^6 cells, having been stored in ethanol, were spun and the ethanol was drained off. The pellet was resuspended in approx. 5 ml. of 0.5N-HClO₄ at 0°, and allowed to stand for 10 min. After the suspension had been spun, the HClO₄ was drained off and the pellet digested with 0.5 ml. of 0.3N-NaOH at 37° for 50 min. After the solution had cooled, 0.5 ml. of N-HClO₄ was added and the precipitate was left to flocculate at 0°; the suspension was then spun. The supernatant was carefully pipetted off for readings of E_{260} in micro-cuvettes. Samples of the supernatant were also used for the orcinol determination of RNA and for radioactivity counting.

The supernatant (1.0 ml.) from 10^6 freshly isolated cells gave $E_{260}^{1\text{cm.}} 0.1$. An approximation to the quantity of RNA that this represents was obtained by using a value of 34 for $E_{260}^{1\text{cm.}}$ at 260 mμ for hydrolysed RNA (Scott *et al.* 1956). No material that reacted with diphenylamine was found in the RNA fraction. The E_{260} value increased linearly with increasing cell number. E_{260}/E_{280} ratios 1.31–1.45 and E_{260}/E_{240} ratios 1.7–2.1 were obtained at various times of culture. In the absence of PHA, E_{260}/E_{240} ratios were generally low. One $E_{260}^{1\text{cm.}}$ unit of phenol-purified lymphocyte RNA, at pH 5.2, produced approx. 1.3 $E_{260}^{1\text{cm.}}$ units of hydrolysed RNA with an E_{260}/E_{280} ratio 1.33 and an E_{260}/E_{240} ratio of 2.2 when measured under these conditions.

For the measurement of RNA by reaction with orcinol (Kerr & Seraidarian, 1945), 1 vol. of the above-mentioned alkaline-digest supernatant was added to 1 vol. of the orcinol reagent and heated at 100° for 30 min., and the E_{660} value was then read. A ribose standard was used; the amount of supernatant RNA or hydrolysed pure RNA that gave $E_{660}^{1\text{cm.}} 1.0$ produced the same $E_{660}^{1\text{cm.}}$ as 9–12 μg. of ribose. This relationship was found at all times during culture, both with and without PHA (Fig. 1a). A time-course of the release from cell pellets by alkali of material that absorbed at 260 mμ, material that reacted with orcinol and radioactivity previously incorporated from [³H]-uridine showed all three parameters to rise together and to reach a plateau after 30 min.

Measurement of DNA. After the removal of RNA by alkaline digestion, the pellet was washed once with 0.5N-HClO₄ at 0° and spun at 1500g for 4 min. Then 1 ml. of 0.5N-HClO₄ was added and the sample was shaken at 70° for 15 min. before being rapidly cooled in ice. Samples of the supernatant were taken for the measurement of $E_{260}^{1\text{cm.}}$, for reaction with diphenylamine (Burton, 1956) and for ³H determination.

The length of the period of alkaline digestion did not influence the DNA yield. A linear relationship was found between DNA yield and cell numbers. The supernatant (1.0 ml.) from 10^6 freshly isolated cells gave $E_{260}^{1\text{cm.}}$ approx. 0.16; E_{260}/E_{280} ratios 1.4–1.6 and E_{260}/E_{240} ratios 1.2–1.4 were obtained. A uniform relationship between E_{600} after reaction with diphenylamine and E_{260} could not be clearly demonstrated at different times during culture, but the general pattern of DNA change was the same when measured by the two methods (Fig. 1b).

A time-course of the release by hot HClO₄ of DNA from [³H]thymidine-labelled lymphocytes showed that, after a

rapid phase for the first 10 min., the concentration of DNA hydrolysis products then continued to rise at a lower rate. E_{260} , material reacting with diphenylamine and radioactivity were in agreement except that the two last-named parameters showed a lag over the first few minutes as the temperature rose. This probably reflects the early liberation of purine residues (Hodes & Chargaff, 1956). In an experiment with purified calf-thymus DNA a similar lag was found, but the increase in E_{260} was complete in 10 min. With pig lymphocyte cultures, the release of DNA-hydrolysis products did not completely stop in an hour, though this effect was less marked with freshly prepared cultures.

RESULTS

Appearance of cultures. In cultures without PHA, the cells remained as a small smooth sediment at the bottom of the culture vessels. On the first day after the addition of PHA (0.6 mg./ml. of serum) the cells were spread out as a thin film adhering to the glass, and vigorous shaking was required to dislodge them. On the second day the cells were easily suspended as free granular clumps. With excess of PHA (3 mg./ml. of serum) these effects of adherence and clumping were still noted.

Quantitative nucleic acid changes during culture. Fig. 1 shows the nucleic acid changes found when

Fig. 1. Changes in the concentrations of RNA (a) and of DNA (b) during culture of pig lymphocytes in the presence or absence of PHA (0.6 mg./ml. of serum). Approx. 5×10^6 cells were grown at 37° in 5 ml. of medium (25% of autologous serum, 67% of medium 199 and 8% of 0.14 M-NaCl) under a gas phase of air + CO_2 (19:1). $\bullet$, E_{260} with PHA; $\circ$, E_{260} without PHA; $\blacksquare$, appropriate sugar reaction (orcinol for RNA, diphenylamine for DNA) with PHA; $\square$, appropriate sugar reaction without PHA.

pig lymphocytes (10^6 cells/ml.) were cultured in 25% serum for 4 days with or without PHA (0.6 mg./ml. of serum). In the absence of PHA, nucleic acid concentrations fell. In the presence of PHA, the total RNA content of cultures doubled by the end of the second day and fell by the fourth day; sometimes this fall occurred in the third day. At cell concentrations of 2×10^6 cells/ml., some factor limited the increase in RNA content early on the second day. In the presence of PHA, the DNA content rose after a day of culture. The E_{260} value for DNA fell for the first day and then rose to its original value. This fall on the first day sometimes exceeded that in unstimulated cultures. The subsequent rise in E_{260} sometimes did not occur, although the value always exceeded that in unstimulated cultures at times of more than 1 day. In the presence of PHA, RNA/DNA ratios were low on the fourth day. In cultures of human lymphocytes, nucleic acid changes were often slower than in cultures of pig lymphocytes.

Variation of response with PHA concentration. Fig. 2 shows the RNA content of cultures of pig lymphocytes grown for different times in the presence of various concentrations of PHA. The serum concentration was constant at 25%. The PHA concentration is expressed as mg. of PHA/ml. of serum. At very low PHA concentrations there was no response; indeed, a slightly accelerated fall in E_{260} of both RNA and DNA was consistently found at such PHA concentrations. After a response over a broad range of PHA concentrations on the first day of culture, a further response on the second day occurred only over a restricted range of concentrations. In several experiments a sharp optimum response was demonstrable at this time with PHA concentrations of about 0.6 mg./ml. of serum. After the second day, the total RNA content declined, with an accelerated breakdown occurring near optimum PHA concentrations. The optimum PHA concentration for human

Fig. 2. Total RNA concentration in cultures of pig lymphocytes (10^6 cells/ml.) grown for different times in 25% serum with various concentrations of PHA. Each point represents the mean of duplicates of 2×10^6 cells. $\circ$, 22 hr. culture; $\bullet$, 46 hr. culture; $\square$, 66 hr. culture.

cultures was the same as, or slightly less than, that for pig cultures. Inhibition by an excess of PHA was also found when preparations of partially purified PHA were used.

Variation of response with serum concentration. Fig. 3 shows two experiments in which pig cells were grown for 2 days with various concentrations of PHA, in the presence of different concentrations of serum (5–40%). The PHA concentration is expressed as mg. of PHA/ml. of total medium. As the serum concentration was raised, the amount of PHA required for an optimum response increased. Fig. 4 shows a similar series of curves (5–75% serum) in which the PHA concentration was expressed as mg. of PHA/ml. of serum. On the ascending part of the curves the response was proportional to the PHA/serum ratio. Thus cultures containing 75% of serum required 15 times as much PHA (absolute quantity) as was needed to produce the same response in cultures containing 5% of serum. The same result was obtained with partially purified PHA (Difco Laboratories). Similar experi-

ments showed that the initial RNA increase for a given number of cells was not demonstrably dependent on cell concentration, in the range 0.5×10^6 – 2.0×10^6 cells/ml. (Fig. 5).

DISCUSSION

Purity of lymphocyte population. Although a varying amount of contamination by polymorphonuclear leucocytes was present in most of the

Fig. 3. Variation of total RNA concentration after 45 hr. of culture, with PHA concentration, at different serum concentrations. In two separate experiments 3×10^6 pig cells (a) and 1.5×10^6 pig cells (b) were grown at a concentration of 10^6 cells/ml. of medium containing 5% (●), 25% (○), or 40% (□) of autologous serum.

Fig. 4. Variation of total RNA concentration after 48 hr. of culture, with PHA/serum ratio, at three different serum concentrations. 10^6 pig lymphocytes were grown in 1 ml. of medium containing 5% (●), 25% (○), or 75% (□) of autologous serum.

Fig. 5. Variation of total RNA concentration/ 2×10^6 pig cells, after 50 hr. of culture, with PHA/serum ratio and cell concentration, at a constant serum concentration of 25%. ●, 0.5×10^6 cells/ml.; ○, 1.0×10^6 cells/ml.; □, 2×10^6 cells/ml. (At the last-named cell concentration, with the optimum PHA/serum ratios, culture conditions limit RNA increase early on the second day.)

cultures, there is evidence that the transformed cells are derived from lymphocytes (Marshall & Roberts, 1965; Yoffey *et al.* 1965). There is also evidence that, in one species, PHA decreases the activity of polymorphonuclear cells (Pogo, Allfrey & Mirsky, 1966). The accelerated fall in the E_{260} for DNA, produced in some PHA-stimulated cultures at 24 hr., could reflect an increased destruction of these cells. However, Cooper & Rubin (1965), using 98% pure lymphocytes, also reported a fall in the quantity of material absorbing at 260 m μ that can be extracted from cultures with perchloric acid after the addition of PHA. Blood lymphocytes are known, on the basis of various physiological criteria, to consist of at least two major populations (Ottesen, 1954; Everett, Caffrey & Rieke, 1964; Elves, Gough & Israels, 1966), and there is some morphological evidence for the presence of a PHA-responsive lymphocyte population (approx. 70% of the total) and a PHA-non-responsive lymphocyte population (Schrek & Batra, 1966). The enlargement of a population of lymphocytes into macrophages, which do not have a high RNA content (Gough & Elves, 1966), would probably not be detected by the methods used in this work.

Adherence to glass. Goddard & Mendel (1929) showed that a crude PHA preparation causes erythrocytes suspended in a salt solution to adhere to glass. PHA has also been shown to facilitate the binding of lymphoid cells to the surface of cultured cell monolayers in the presence of serum (Moller & Moller, 1965; Holm & Perlmann, 1965); provided that allogeneic combinations of cells were used, lysis of the monolayers occurred. The amount of lysis was proportional to the percentage of blast cells that developed (G. Holm, personal communication). In a similar system, Ginsberg & Sachs (1965) noted that lymphoblastic transformation and monolayer lysis slowly developed in the absence of PHA. It is possible that, at the molecular level, glass presents a surface with an extremely wide spectrum of 'foreign' shapes (antigenic determinants). It is possible that PHA affords a close approximation to such a surface and thus activates the wide spectrum of specific immunologically competent lymphoid cells believed to be continuously circulating in blood (Forsdyke, 1966c, 1967).

Value of nucleic acid changes as an index of both transformation and culture ecology. Growth of cells should continue until it is limited by the concentration of one or more nutrient growth-factors or toxic metabolic products, or until influenced by some intrinsic regulatory mechanism. As shown by RNA concentrations, with 10^6 pig cells/ml. and an optimum PHA concentration this point was not reached until the end of the second day of culture (Fig. 1). The lower rate of nucleic acid increase in human cultures compared with that in pig cultures

may reflect the metabolic state of such cultures or indicate that a smaller number of cells are responding.

DNA concentrations increased on the second and third day of culture, following the development of the capacity of cells to incorporate labelled thymidine (Mueller & Le Mahieu, 1966). This increase probably reflects both the concentration of nutrients available at the time and the stimulation of one population of cells accompanied by the degeneration of others.

Variation of response with PHA concentration. Robbins (1963) reported that as the PHA concentration was increased an increasing percentage of cells transformed. Thus at optimum PHA concentrations many cells transform and only a few are left to degenerate. Since measurement of total RNA present in cultures provides an index of both cell death and transformation, the shape of dose-response curves is likely to be exaggerated by these factors. The inhibition at high PHA concentrations appeared after a day of culture, at a time when cells had detached from glass and were starting to synthesize DNA. If the degree of clumping were higher at high PHA concentrations, further transformation might be prevented by a contact inhibition effect (Eagle, 1965). Other possible explanations are considered elsewhere (Forsdyke, 1966c).

Dependence of response on the PHA/serum ratio. The dependence of the response on the PHA/serum ratio may be demonstrated over the first few hours of culture by using the incorporation of [3 H]uridine as an index of cell activation (D. R. Forsdyke, unpublished work), as well as by measuring the quantitative RNA increase in cultures at later times. There are four simple explanations for this dependence: (i) PHA removes a serum inhibitor; (ii) PHA forms a complex with serum that then activates cells; (iii) PHA and a serum factor are required to act together in order to activate cells; (iv) PHA reacts separately and concomitantly with both serum and cells, but serum PHA-binding sites are in considerable excess.

In terms of the first explanation, no reaction of PHA with cells seems necessary. It is possible that a molecular species, present in serum, that blocks all the potential antigenic sites at the surface of glass might be inactivated by PHA. On the other hand, the equilibrium of the free and cell-bound forms of an inhibitory molecule might be influenced by PHA, or some vital growth factor might be displaced from a complex with another serum molecule. The other explanations involve a direct reaction of unknown duration of PHA with cells. Such explanations would be in keeping with reports of the passive adsorption of transforming activity from PHA by lymphoid cells (Kolodny & Hirschhorn, 1964; Borjeson, Chessin & Welsh, 1966).

Extracts of *Phaseolus vulgaris* beans are known to react with serum α_2 -globulins (Beckman, 1962) and a range of other serum components (Holland & Holland, 1965). Haemagglutination induced by a partially purified preparation of PHA is inhibited by a serum mucoprotein (Steck & Wallach, 1965). In preliminary experiments to test the hypothesis that the PHA requirement is proportional to macroglobulin concentrations (Forsdyke, 1966c), human cells were grown in newborn serum poor in macroglobulin (Franklyn & Kunkel, 1958), or in pathological serum containing macroglobulins. No alteration of the PHA requirement for an optimum response was detectable. Addition of a macroglobulin fraction prepared from normal serum only weakly influenced the PHA requirement.

Many of the observations presented both in this paper and by others (Cooper & Rubin, 1965; Fisher & Mueller, 1967) would be unified if two properties of the serum factor were (a) a degree of solubility in perchloric acid producing interference with E_{240} and E_{280} and hence with nucleic acid measurement, and (b) an affinity for cells, or the glass of culture vessels, that is inhibited by PHA.

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