Incorporation of [5-³H]Uridine and Attachment of Cells to Glass during Activation of Lymphocytes Induced by Phytohaemagglutinin

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1. Shortly after the addition of phytohaemagglutinin to cultures, partially purified pig blood lymphocytes showed increased labelling of RNA by [5-³H]uridine and began to attach to the glass of the culture tubes. 2. In the presence of phytohaemagglutinin most cells became attached to glass during the first 5 hr. of culture; those first attaching had a low mean RNA/DNA ratio. In the absence of phytohaemagglutinin cell attachment to glass was diminished. 3. During the first 5 hr. of culture the rates of increase of radioactivity/unit of DNA of the attached and unattached populations in the presence of phytohaemagglutinin were of the same order and exceeded that of cultures without phytohaemagglutinin. 4. The increase of labelling in cultures to which phytohaemagglutinin was added after cells had sedimented to glass was greater than that occurring in cultures in which cells were resuspended before phytohaemagglutinin was added.

Usually when blood lymphocytes are cultured *in vitro* they degenerate (Trowell, 1965). However, when PHA† is added to cultures the cells are activated to transform and divide (Nowell, 1960). This phenomenon offers an opportunity to examine mammalian cellular control mechanisms in a relatively simple primary explant system *in vitro*. Since blood lymphocytes are in suspension phase *in vivo*, and may be isolated by very gentle procedures, their transfer to an environment *in vitro* seems likely to be less traumatic than the transfer of more organized tissues.

The following sequence of changes has been described in cultures at various times after the addition of PHA. In the first few hours there are an interaction with a serum factor of possible cationic acid-soluble nature (Forsdyke, 1967*b*, 1968; Agrell & Karlsson, 1967), cellular histone changes (Killander & Rigler, 1965; Pogo, Allfrey & Mirsky, 1966), increased labelling of RNA with radioactive precursors, which may partly reflect the synthesis of a new RNA species (Cooper & Rubin, 1965; Mueller & Le Mahieu, 1966; Forsdyke, 1968), and cells begin to attach to the glass of the culture vessels (Killander & Rigler, 1965; Forsdyke, 1967*a*). After several hours of culture, attachment to glass becomes complete, enhanced labelling of ribosomal precursor RNA becomes evident (Rubin & Cooper, 1966; Kay, 1966; Forsdyke, 1968) and

activation of cells becomes irreversible (Mellman & Rawnsley, 1966). On the second day of culture, cells initiate DNA synthesis and cell division, detach from glass, and become inhibited if excess PHA is present (Forsdyke, 1967*b*).

In order to discover which of these events are critical to the transformation process and how they are interrelated in molecular terms, it is first necessary to distinguish them from events concomitant with, but not essential for, transformation; such events include those normally occurring when cells are taken from an environment *in vivo* to one *in vitro*, and those secondary to the active metabolism of PHA-activated cells (Forsdyke, 1968). The present paper describes studies of the relationship between the increasing attachment of cells to glass and the enhanced labelling with [³H]uridine occurring during the first few hours of culture with PHA. The work is presented more fully elsewhere (Forsdyke, 1967*c*).

MATERIALS AND METHODS

Details of materials and of methods of preparation and culture of pig blood lymphocytes have been given previously (Forsdyke, 1967*b*, 1968).

Resuspension of sedimented cells. Cultures, of 1 ml. volume in soda-glass round-bottomed centrifuge tubes of 15 ml. total capacity and 1.4 cm. internal diameter, contained 10⁶ cells and 1 μ C of [5-³H]uridine. In the four experiments performed 80%, 80%, 70% and 70% of the nucleated cells present in culture were lymphocytes. The tubes were briefly gassed with air+CO₂ (19:1), stoppered

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† Abbreviation: PHA, phytohaemagglutinin.

and left for 1 hr. at 37° for the cells to sediment. Then one series of tubes was gently shaken to resuspend the cells. The nozzle of a microsyringe was directed below each culture surface and 8 μ l. of 0.14M-NaCl containing 40 μ g. of 'PHA-P' (Difco Laboratories, Detroit, Mich., U.S.A.) was gently squirted into the medium in a manner designed to ensure mixing, but not to resuspend cells off the bottom in tubes that had not been shaken. To control tubes was added 8 μ l. of 0.14M-NaCl without PHA. The cultures were again gassed and stoppered, and incubation continued. At various times cultures were harvested for the determination of ^3H in acid-precipitable material (Forsdyke, 1968).

Separation of 'attached' from 'unattached' cell populations. In the three experiments performed, 60%, 65% and 70% of the nucleated cells present were lymphocytes. Approx. 300ml. of freshly isolated cells ($3 \times 10^6/\text{ml.}$) in autologous serum-0.14M-NaCl (3:1, v/v) was incubated in a 500ml. glass flask at 37° with periodic agitation to keep the cells suspended. At 10min. intervals, samples (11ml.) were pipetted into 50ml. silicone-treated glass flasks containing 22ml. of culture medium 199 at 37°. After addition of [^3H]uridine (1–2 $\mu\text{C}/\text{ml.}$ of culture), samples (4ml.) were immediately pipetted into warm centrifuge tubes with the characteristics described above. Then 12 μ l. of 0.14M-NaCl containing 0.6mg. of crude PHA (Burroughs Wellcome and Co., London, N.W. 1) was gently squirted into each culture; 12 μ l. of 0.14M-NaCl was added to control tubes not having PHA. The tubes were briefly gassed, sealed with silicone-rubber bungs and incubated at 37°.

Cultures were harvested in the reverse order of their setting up. Cells in control cultures with and without PHA were pelleted by centrifugation (1500g for 2min.) and the supernatants discarded. The cells were washed once by resuspension in 0.14M-NaCl at 0° and were finally stored in ethanol at –10°.

To separate 'attached' from 'unattached' cells, the culture fluid containing suspended cells was poured off with one movement into a fresh centrifuge tube. Remaining unattached cells were washed out by gently pipetting 4ml. of 0.14M-NaCl at 0° down the same side of the tube as the culture fluid had been poured off from, and then, after a few seconds, pouring it off again. A second wash of 4hr. cultures brought no more 'attached' cells off into the 'unattached' fraction. However, at earlier times cells were less firmly bound. The attached cells were fixed in ethanol at –10°. Unattached cells were pelleted by centrifugation, washed once and fixed in ethanol.

Details of methods of nucleic acid extraction, assay and counting have been presented previously (Forsdyke, 1967b, 1968). RNA hydrolysis products in acid solution (0.6ml.) were added to 10ml. of aqueous scintillator (Evans, 1966) for determination of radioactivity. The efficiency of counting was 4%. The sum of the radioactivities in attached and unattached cell fractions was generally the same as, or slightly less than, that found in corresponding control cultures containing both attached and unattached cells. The sum of E_{260} nucleic acid estimations generally slightly exceeded that of the control cultures. E_{240} values when summed exceeded those of the controls even more. The sum of diphenylamine E_{600} DNA values was generally the same as, or slightly less than, controls. The nature of substances of possible serum origin that interfere with nucleic acid assays and produce lowered E_{260}/E_{240} ratios has been discussed previously (Forsdyke, 1967b,c).

Time before and after shaking and adding PHA (min.)

Fig. 1. Effect of resuspending sedimented cells on the enhanced incorporation of [^3H]uridine ('activation') induced by PHA. Cultures (1ml.) containing 10^6 cells, 1 μC of [^3H]uridine (26 $\text{C}/\text{m-mole}$) and 25% of autologous pig serum were incubated for an hour at 37°, and then one series of tubes was gently shaken. PHA (40 $\mu\text{g.}$ of Difco 'PHA-P') was immediately added and incubation continued. At intervals cultures were harvested for determination of acid-precipitable radioactivity. For further details see the Materials and Methods section. ○, Unshaken, no PHA; □, shaken, no PHA; ●, unshaken, + PHA; ■, shaken, + PHA.

RESULTS

Resuspension of sedimented cells. Cells were preincubated for 1 hr. with [^3H]uridine, and then one series of tubes was gently shaken to resuspend sedimented cells. PHA was then added and the incubation continued. In control cultures without PHA the time-course of labelling of acid-precipitable material was approximately linear; the experimental points could be extrapolated back to zero radioactivity at zero time (Fig. 1), and shaking did not appreciably affect this time-course. Cultures with PHA showed increased labelling; those that had not been shaken showed a 20% (s.e.m. $\pm 7\%$) greater degree of labelling within 1–2 hr. after addition of PHA than cultures with PHA that had been shaken (four experiments; $P=0.02$ by Wilcoxon signed ranks test; Siegel, 1956).

Attachment to glass. Cells were incubated with [^3H]uridine in the presence or absence of PHA. By pouring off the culture fluid containing suspended cells at various times of culture it was possible to divide the cells into two populations, those attached to glass and those unattached. RNA and DNA

Table 1. Comparison of the properties of attached and unattached populations of cells after incubation for 5 hr. with [5-³H]uridine in the presence or absence of PHA

The 4 ml. cultures contained approx. 4×10^6 cells, $8\mu\text{C}$ of $[5\text{-}^3\text{H}]\text{uridine}$ ($13\text{C}/\text{m-mole}$), 25% (v/v) of autologous pig serum and 0.6 mg. of PHA (Burroughs Wellcome crude preparation). To separate 'attached' from 'unattached' cells, the culture fluid containing suspended cells was poured off into a fresh centrifuge tube (tube no. 3 into tube no. 5, and tube no. 4 into tube no. 6). Remaining unattached cells were washed out with 4 ml. of 0.14M-NaCl at 0° . After storage of cell pellets in ethanol, RNA was extracted with 0.3N-NaOH , and DNA with 0.5N-HClO_4 , as described by Forsdyke (1967*b*). Extracted RNA was determined by $E_{260}^{1\text{cm.}}$ measurement, and the radioactivity incorporated was determined in aqueous scintillator. Extracted DNA was determined by $E_{260}^{1\text{cm.}}$ measurement and by reaction with diphenylamine reagent: 1 vol. of extract was added to 2 vol. of reagent and, after incubation at 30° for approx. 16 hr., $E_{800}^{1\text{cm.}}$ was measured (Burton, 1956). Results are expressed as if all extracted material were in a total volume of 1 ml.

		RNA					DNA*				
	Tube no.	$E_{260}^{1\text{cm.}}$	E_{260}/E_{240}	E_{260}/E_{280}	Counts/min./ $E_{260}^{1\text{cm.}}$	RNA/DNA*	$E_{260}^{1\text{cm.}}$	E_{260}/E_{240}	E_{260}/E_{280}	$E_{800}^{1\text{cm.}}$	Counts/min./ $E_{800}^{1\text{cm.}}$
PHA present											
Control	1	0.190	(1.86 1.30)		66000	1.15	0.660	(1.41 1.32)		0.165	76000
	2	0.192	(1.77 1.31)		67500	1.25	0.603	(1.39 1.31)		0.153	84700
Attached	3	0.082	(1.36 1.17)		82800	0.91	0.465	(1.29 1.31)		0.090	75300
	4	0.090	(1.50 1.21)		84200	1.00	0.378	(1.43 1.26)		0.090	84200
Unattached	5	0.108	(1.74 1.28)		48800	1.33	0.324	(1.33 1.25)		0.081	65000
	6	0.142	(1.42 1.26)		36400	1.90	0.324	(1.30 1.25)		0.075	69000
PHA absent											
Control	1	0.168	(1.47 1.09)		52300	1.13	0.606	(1.40 1.29)		0.151	58700
	2	0.184	(1.73 1.27)		49400	1.20	0.657	(1.38 1.32)		0.153	59300
Attached	3	0.032	(0.88 1.06)		37200	—	0.156	(1.10 1.26)		0.003	—
	4	0.016	(0.72 0.80)		52200	—	0.099	(1.10 1.17)		0.000	—
Unattached	5	0.154	(1.79 1.26)		46200	1.40	0.507	(1.44 1.33)		0.111	64000
	6	0.164	(1.86 1.26)		45800	1.20	0.549	(1.40 1.35)		0.135	55600

* Since a consistent relationship between $E_{260}^{1\text{cm.}}$ and $E_{600}^{1\text{cm.}}$ diphenylamine determination of DNA has not been established (Forsdyke, 1967c), both values are recorded. $E_{600}^{1\text{cm.}}$ values were used for the calculation of the RNA/DNA and counts/min./unit of DNA ratios. A similar qualitative pattern of ratios was obtained when $E_{260}^{1\text{cm.}}$ values were used.

hydrolysis products were separately extracted from cells and estimated by measurement of E_{260} or by reaction with diphenylamine (Forsdyke, 1967b). In addition the ³H in the RNA fraction was estimated.

Table 1 shows representative data obtained at a single time-point, in this case 5 hr. after the initiation of culture. The following observations were made. (a) More cells attached to glass in the presence of PHA than in the absence of PHA. (b) The attached population had a lower mean RNA/DNA ratio than the control cultures; the unattached population had a higher mean RNA/DNA ratio than the control cultures. (c) E_{260}/E_{240} and E_{260}/E_{280} ratios of the extracted RNA and DNA fractions were usually low in attached populations, and this was especially marked in the absence of PHA. (d) The mean specific radioactivity of RNA was highest in the attached population in the presence of PHA; the specific radioactivity of RNA in the unattached population in the presence of PHA was less than that of control cultures with PHA. (e) In the presence of PHA the radioactivities in both attached and unattached

populations, when expressed as counts/min./unit of DNA, were of the same order and exceeded those of control cultures without PHA.

Fig. 2 shows a time-course of the process of cell attachment as measured by the increase in attached DNA and decrease in unattached DNA. A high proportion of the cells became attached over 5 hr. of culture in the presence of PHA. Within the time-period examined no appreciable change in the total RNA and DNA content of cultures was induced by PHA. No change in the RNA/DNA ratio of the attached population could be demonstrated over 5 hr. of culture. The RNA/DNA ratio of the unattached population rose slightly during this period.

Fig. 3 shows the change in specific radioactivity of total RNA occurring over the first 4 hr. of culture in attached and unattached populations in the presence of PHA, and in control cultures in the presence or absence of PHA. In control cultures specific radioactivity of total RNA rose linearly with time. The specific radioactivity of the RNA of the attached population of cells also rose linearly

Fig. 2. Attachment of cells to glass in the presence of PHA. Cultures (4ml.) containing approx. 4×10^6 cells were incubated either with or without PHA (0.6 mg. of Burroughs Wellcome crude PHA) in 25% (v/v) autologous pig serum. At intervals the culture fluid containing suspended cells was poured off into a fresh tube. Remaining unattached cells were washed out with 4 ml. of 0.14 M-NaCl. The DNA contents of cells remaining in the original tubes ('attached'), and of cells poured off ('unattached'), were determined. For further details see Table 1 and the Materials and Methods section. $\circ$, Control cultures, no PHA; $\bullet$, control cultures, + PHA; $\square$, unattached cells, no PHA; Δ , attached cells, no PHA; $\blacksquare$, unattached cells, + PHA; $\blacktriangle$, attached cells, + PHA. Each point is the mean of duplicates.

Fig. 3. Change in specific radioactivity of RNA of attached and unattached populations of cells in the presence of PHA. Cultures contained $[5\text{-}^3\text{H}]\text{uridine}$ ($1 \mu\text{C}/\text{ml.}$ of culture; $26 \text{ c}/\text{m-mole}$). Other details are as given in Table 1 and Fig. 2. $\circ$, Control cultures, no PHA; $\bullet$, control cultures, + PHA; $\square$, unattached cells, no PHA; $\blacksquare$, unattached cells, + PHA; Δ , attached cells, no PHA; $\blacktriangle$, attached cells, + PHA.

Fig. 4. Change in RNA radioactivity, expressed per unit of DNA, of attached and unattached populations of cells in the presence of PHA. Cultures contained $[5\text{-}^3\text{H}]\text{uridine}$ ($2 \mu\text{C}/\text{ml.}$ of culture; $26 \text{ c}/\text{m-mole}$). Other details are as given in Table 1 and Fig. 2. $\circ$, Control cultures, no PHA; $\bullet$, control cultures, + PHA; $\square$, unattached cells, no PHA; $\blacksquare$, unattached cells, + PHA; Δ , attached cells, no PHA; $\blacktriangle$, attached cells, + PHA.

over 4 hr. of culture. However, the specific radioactivity of the RNA of the unattached population was not linear and the rate of increase became less over 4 hr. of culture.

Fig. 4 shows a similar experiment to that shown in Fig. 3. Here the radioactivities incorporated are expressed as counts/min./unit of DNA. The values for attached and unattached cells in the presence of PHA were in close agreement with those for control cultures with PHA, and all exceeded the values found in control cultures without PHA.

DISCUSSION

Uridine incorporation, activation and irreversible activation. Evidence has been presented suggesting that, for some hours after the addition of PHA, activation of cells can be reversed by removing free PHA from the medium (Mellman & Rawnsley, 1966). At optimum PHA concentrations [0.6 mg. of PHA (Burroughs Wellcome)/ml. of serum], a 2 hr. period of exposure of cells to PHA permits only a small degree of transformation to follow, although higher PHA concentrations are more effective (Forsdyke, 1967c). After activation, transforma-

tion is independent of the presence of PHA in the medium until a second PHA-dependent activation point is reached some days later (Mellman & Rawnsley, 1966; Yamamoto, 1966; Ling & Holt, 1967; Forsdyke, 1967c). The extent to which the early increase in uridine incorporation induced by PHA represents irreversible activation, or simply the removal of some 'safety catch' from cells, is uncertain. The time of the initiation of the synthesis of predominantly ribosomal RNA on the first day of culture (Rubin & Cooper, 1966; Kay, 1966; Forsdyke, 1966a,b, 1968) could represent an irreversible activation point.

Attachment to glass. When confirming the early work of Goddard & Mendel (1929) that PHA causes erythrocytes to attach to glass, McIntyre & Ebaugh (1962) noted that leucocytes were also attaching. Killander & Rigler (1965) noted that leucocytes attached to glass slides within 1 hr. of the addition of PHA to cultures. Lucas (1967), using lymphocytes of 98% purity, reported a 25% decrease in the number of cells that could be resuspended during the first 24 hr. of culture, but did not explain this observation on the basis of attachment to glass.

It is not known whether the PHA requirement for an optimum degree of attachment is the same as that for optimum transformation, nor whether the factor or factors in PHA causing transformation are the same as those causing attachment. The effect could relate to the well-known phenomenon of cell adherence to glass found with various primary explant cultures and propagated tumour-cell lines (Lieberman & Ove, 1958; Eagle, 1965); this in turn could relate to charge differences between the cell surface and glass (Vassar & Culling, 1964).

In this discussion it will be shown that the present experiments provide further evidence for PHA-induced lymphocyte attachment to glass, but suggest that such attachment plays no fundamental role in cell activation, when activation is defined solely as an early increase of uridine incorporation.

The finding of greater PHA-induced labelling in cultures that had not been shaken (Fig. 1) is compatible with the proposition that attachment to glass is a necessary concomitant of cell activation. However, the shaking of cultures may have a number of effects besides increasing the time for cells to become attached to glass. For example, cells would be separated from each other so that opportunities for cell-cell interaction would be decreased; the microclimate created by the metabolism of cells in close proximity would probably be altered; the rapid mixing of PHA added shortly after the cultures had been shaken would be facilitated.

Kinetics of attachment (Fig. 2). Suspensions of approx. 4×10^6 cells were pipetted into round-

bottomed tubes of internal dimensions such that the maximum distance a cell had to travel when sedimenting to glass was 2-3 cm. The total area on which cells could settle was approx. 3 cm^2 . If the average area occupied by a cell is $100 \mu^2$, and if initially cells stick only to glass and not to each other, 3 cm^2 should maximally accommodate approx. 3×10^6 cells. Thus the kinetics of attachment shown in Fig. 2 could be influenced by factors such as the time of cells to sediment and cell competition for binding sites on glass. The data give no information on the maximum number of cells that can attach, but do show that this is a function of most of the cells present in culture. In that most of the cells present are lymphocytes, it is likely that they are included among the attached cells.

Differences between attached and unattached populations. Clearly the extent to which the population data of this paper can be interpreted in terms of individual cells depends on a correlation with morphological and radioautographic evidence provided by light-microscopy (Cooper & Rubin, 1965; Hayhoe & Quaglino, 1965; Darzynkiewicz, Krassowski & Skopinska, 1965; Winter & Yoffey, 1966). As discussed by Forsdyke (1967b), there is evidence of heterogeneity among the cell population referred to as small lymphocytes, and in addition cultures contained polymorphonuclear cells and a few large lymphoid cells. On purely physical grounds it is likely that the smallest and most dense cells in culture would sediment to glass first. Small lymphocytes, as judged by their isopycnic position in sucrose density gradients, are denser than other lymphoid cells (D. R. Forsdyke, unpublished work). With their small rim of cytoplasm they might be expected to have a lower RNA content than other lymphoid cells. The low RNA/DNA ratio of attaching cells (Table 1) is compatible with the proposition that small lymphocytes attach to glass first.

The specific radioactivity of the attached population rose in a linear manner during the first 4 hr. of culture (Fig. 3) and no rise in the RNA/DNA ratio of that population could be demonstrated in that time. This shows that, in these respects at least, the population is uniform. If attaching cells removed more radioactivity from the population of unattached cells than RNA, then the radioactivity/unit of RNA of the unattached population would fall. Thus the data of Fig. 3 do not show that the attached population has a higher mean rate of uridine incorporation/cell or that the unattached population has a lower mean rate of uridine incorporation/cell. When incorporation data were presented as radioactivity/unit of DNA it was found that the mean incorporation rate appeared to be stimulated equally by PHA in both attached

and unattached populations (Fig. 4). Assuming that all cells have the same DNA content (Hale & Wilson, 1959), it would appear that there is an increase in the mean incorporation/cell of unattached cells before the attachment of many of these cells to glass. The extent to which this can be taken as showing that cells are activated by PHA before they attach to glass depends on the extent to which the cells in culture can be considered as a uniform population. For example, a population of cells with a rate of uridine incorporation lower than that of control cultures without PHA and possessing a low RNA/DNA ratio might become attached to glass together with another population that was activated by such attachment; then the counts/min./unit of DNA of the unattached population would be elevated (since more DNA than radioactivity would be removed from the unattached population), and the increased counts/min./unit of DNA of the glass-activated population would be masked. Data presented as counts/min./unit of RNA would not be so dramatically affected. The specific radioactivity of total RNA of the attached population is probably a minimum estimate since, as judged by E_{260}/E_{240} ratios (Table 1), there is some interference with the E_{260} measurement of the total RNA content of these cells (Forsdyke, 1967b).

On balance, the data presented here are considered to show that activation of cells by PHA is independent of their attachment to glass. Three separate lines of evidence may be interpreted as supporting this conclusion. PHA may cause lymphocyte transformation (a) *in vivo* (Gamble, 1966), (b) in millipore chambers implanted in eggs (Owen, 1966) and (c) in cultures containing cells suspended in semi-solid agar phase (Coulson, Turk, Glade & Chessin, 1968).

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