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LYMPHOCYTE TRANSFORMATION *IN VITRO*

I. TISSUE CULTURE CONDITIONS AND QUANTITATIVE MEASUREMENTS

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SUMMARY

The quantitative evaluation of the reactivity in human PHA-stimulated lymphocyte cultures was studied. A technique for the measurement of DNA synthesis by means of thymidine-2-^{14}C incorporation was standardized and its reproducibility determined. Using this method a number of variables in culture conditions were investigated. The results obtained are discussed.

INTRODUCTION

Many investigators have described the phenomenon of human lymphocyte transformation *in vitro* in short-term tissue culture experiments. These changes can be induced by various stimuli, which can be classified in four categories:

(1) Non-specific stimuli such as PHA, SLS, pokeweed mitogen, staphylococcus toxin, etc. (Nowell, 1960; Kolodny & Hirschhorn, 1964; Ling & Husband, 1964; MacKinney, 1964).

(2) Antigens against which the lymphocyte donor is sensitized (Elves, Roath & Israels, 1963; Hirschhorn *et al.*, 1963; Marshall & Roberts, 1963; Pearmain, Lycette & Fitzgerald, 1963; Ling & Husband, 1964).

(3) The presence of lymphocytes of another unrelated donor (Bach & Hirschhorn, 1964; Bain, Vas & Löwenstein, 1964; Bain & Löwenstein, 1964).

(4) Anti-leucocyte antibodies (Gräsbeck, Nordman & de la Chapelle, 1963).

A great deal of work has been performed in this field, but very few experiments were carried out to determine optimal conditions for reproducible results and quantitative measurements. Evaluation of the activity of the lymphocytes has been performed in many different ways. The diversity of methodology makes it difficult to compare the results of different investigations.

This paper deals with the experiments we performed to develop a technique which would enable us to measure the reactivity of the lymphocytes under optimal and reproducible tissue culture conditions. For this purpose only lymphocytes stimulated with PHA were used.

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At first we tried to arrive at a reproducible determination of the reactivity by morphological interpretation of the blastogenesis; 1000 cells/slide were counted and the relative number of transformed cells determined. After a thorough trial it became clear to us that we were not able to get reproducible results with this method if blind counting was performed. This made us decide to try to evaluate the reactivity by the determination of DNA synthesis. For this purpose radioactively-labelled thymidine was added to the culture medium for a certain period and the radioactivity, incorporated by the cells, was determined.

In the development of this technique the following points were investigated:

(1) The correlation between the radioactivity incorporated by the cells and that actually present in the DNA.

(2) The influence of the thymidine concentration in the medium upon the incorporation.

(3) The influence of different periods of incubation with thymidine upon the incorporation.

(4) The thymidine incorporated on the different days of culture.

(5) The reproducibility of the DNA synthesis measurements and the reproducibility of the tissue culture experiments as such.

Thus having acquired a technique for the measurement of reactivity, which fulfils our requirements with regard to reproducibility, the following variables were investigated with the DNA synthesis as parameter in order to determine optimal conditions for lymphocyte transformation by phytohaemagglutinin:

(6) The influence of different media and buffer systems.

(7) The influence of different serum sources and concentrations.

(8) The influence of the presence of granulocytes in the tissue culture.

(9) The influence of different cell concentrations.

(10) The influence of different PHA concentrations.

(11) The number of viable cells present on the different days of culture and their size distribution.

(12) The amount of DNA present on the different days of culture and its relation to the number of cells.

MATERIALS AND METHODS

Preparation of the lymphocyte suspension

Venous blood was drawn in heparin without preservative ('Thromboliquine', Organon, Oss, Holland) with a final concentration of 200 μg/ml.

The granulocytes were removed by filtration of the blood through 'Leukopak' filters (Fenwal Laboratories, Morton Grove, Illinois, U.S.A.) at 37°C. The red cells were sedimented at 37°C for 30–45 min after addition of ¼ volume of 5% Dextran (molecular weight 180·000, N.V. Poviet, Amsterdam). The lymphocyte-rich plasma was removed and centrifuged for 10 min at 400 *g*. The cells were washed twice by re-suspension in Hanks's BSS and centrifugation for 10 min at 260 *g* at room temperature. The cells were then re-suspended in Eagle's Minimum Essential Medium for suspension cultures (Eagle, 1959) without L-glutamine (Grand Island Biology Co., New York, U.S.A.), +20% serum inactivated by heating at 56°C for 30 min, +L-glutamine 0·1 mM/ml, +penicillin 100 U/ml, +streptomycin 100 μg/ml.

Counting of the white cells was performed in duplicate on an electronic cell counter (Coulter Electronics Model A) after lysis of the red cells with Triton X 100 and saponin according to the method of Helleman (1966). The lymphocyte recovery was 30–50%. The

granulocyte contamination was always less than 4% and usually less than 2%. The cells were further resuspended in the above mentioned culture medium to a final concentration of 750,000 lymphocytes/ml.

Lymphocyte culture

If not otherwise mentioned aliquots of 4 ml (3×10^6 lymphocytes) are cultured in screw-capped test tubes (16 × 125 mm, Kimax) at a temperature of 37°C. For stimulation PHA (Burroughs Wellcome) was added, 0·05 ml/tube. If necessary, the pH was corrected daily with 5% sodium bicarbonate or with 0·1 N-HCl.

Treatment of the cells for morphological evaluation

Five hours before harvesting 0·03 μg vinblastine sulphate was added per tube. The cells were treated according to the method of Moorhead *et al.* (1960) and Bach & Hirschhorn (1964).

Determination of thymidine-2-^{14}C incorporation

Varying periods before harvesting of the cells, thymidine-2-^{14}C (Philips Duphar, Petten, Holland) was added in varying amounts and specific activity. The added volume was 0·1 ml. At harvesting the cells were washed twice in ice-cold saline solution, once in ice-cold 2% acetic acid, the sediment was resuspended in 2 ml 2% acetic acid, put in a planchette and dried either in a desiccator or by heating. The radioactivity of the dried material was determined by counting for 10 min in a Geiger–Müller counter (Philips, efficiency 1%).

Isolation and determination of the total DNA content

The method described by McIntire & Sproull (1957) was used. In essence this technique depends on an alkaline hydrolysis of the RNA whereafter the DNA is precipitated by salmine. This precipitate is hydrolysed in 6 N-HCl and the nucleotide content of the lysate measured spectrophotometrically at 260 μ.

The preparation of pure granulocyte suspensions

Granulocyte purification was performed according to Rabinowitz (1964). After Dextran sedimentation of the blood, the leucocyte-rich supernatant was incubated on a siliconized glass bead column for 30 min at 37°C. The granulocytes and monocytes adhere to the glass surface. The lymphocytes can be washed away with serum and Hanks's BSS, whereafter the granulocytes can be eluted with EDTA in low concentrations (disodium EDTA 0·2 g, NaCl 8·0 g, KCl 0·2 g, Na_2HPO_4 1·15 g, KH_2PO_4 0·2 g, and glucose 0·2 g). Granulocyte recovery of 15% with a lymphocyte contamination of less than 5% could always be obtained.

Tissue culture medium

A comparison was made between MEM-S (Gibco) and TC 199 (Gibco). Both media were tested either with the bicarbonate buffer as described originally or with Tris–HCl buffer (final concentration 0·025 M).

Serum

Unless otherwise mentioned the media are supplemented with 20% serum from one of the following sources:

(a) Human serum from people of blood group AB which were tested for irregular red cell isoantibodies and tissue autoantibodies. Negative sera were heat-inactivated and pooled.

(b) Foetal calf serum (Gibco).

(c) Autologous serum.

Determination of the total number of cells present in the culture on the different days and their size distribution

As it is impossible to determine the total number of cells in the culture by conventional methods because of clumping of the cells, a special treatment was introduced to break the clumps and obtain a good cell suspension. The procedure was as follows: The culture tubes were left undisturbed for at least 24 hr in the incubator, whereafter 3·6 ml of the supernatant was removed. The sediment was thoroughly resuspended in the remaining 0·4 ml and 0·1 ml glacial acetic acid was added. After repeated shaking 0·1 ml Cetavlon (cetrimide B.P. 40% w/v I.C.I.) was added and again the mixture was thoroughly shaken. Subsequently 0·1 ml of the suspension was transferred to 50 ml of Ouli's buffered saline, from which dust and other small particles had been removed by filtration through GS filters (0·22 μ pore size, Millipore Corp.).

Distribution curves were made with the Coulter Counter model A at Current Regulation 5, Internal Switch 5 and threshold settings varying from 10–100. From the shape of the curve so obtained it was concluded that the total number of cells could be determined at threshold setting 20. Background activity was determined at the same setting with culture supernatant instead of cells. If A is the reading with cells and B the background, the real number of cells per culture is $(A-B)\times 0{\cdot}6\times 10^3$. The same is true at higher threshold settings for cells bigger than a given size.

RESULTS

The correlation between the total radioactivity incorporated by stimulated lymphocytes and the amount recovered from the DNA

Of four similar experiments, one will be described here in detail. The results of this experiment were consistent with those of the others.

To seven culture tubes with PHA-stimulated cells obtained from a single blood sample 0·6 μCi thymidine-2-^{14}C (specific activity 12·5 mCi/mM) was added after 48 hr of incubation; 24 hr later the procedures shown schematically in Fig. 1, were carried out: After two washings of the cells, tubes Nos. 1, 2 and 3 were handled as described for the determination of total thymidine incorporation. NaOH was added to the sediment of tubes Nos. 4, 5, 6 and 7 and after hydrolysis the contents were diluted 1:10 with distilled water. Half of this volume was separated and the radioactivity estimated. To 0·8 ml of the remaining suspension salmine was added and the radioactivity in the precipitate determined. The recovered radioactivity is also given in Fig. 1. After the necessary volume corrections, the following results can be calculated:

Counts/min	Total sediment	1388	(100%)
	After alkaline hydrolysation	1184	(85%)
	Salmine precipitate (DNA)	1249	(90%)

It becomes clear that 90% of the incorporated thymidine is in fact present in the DNA.

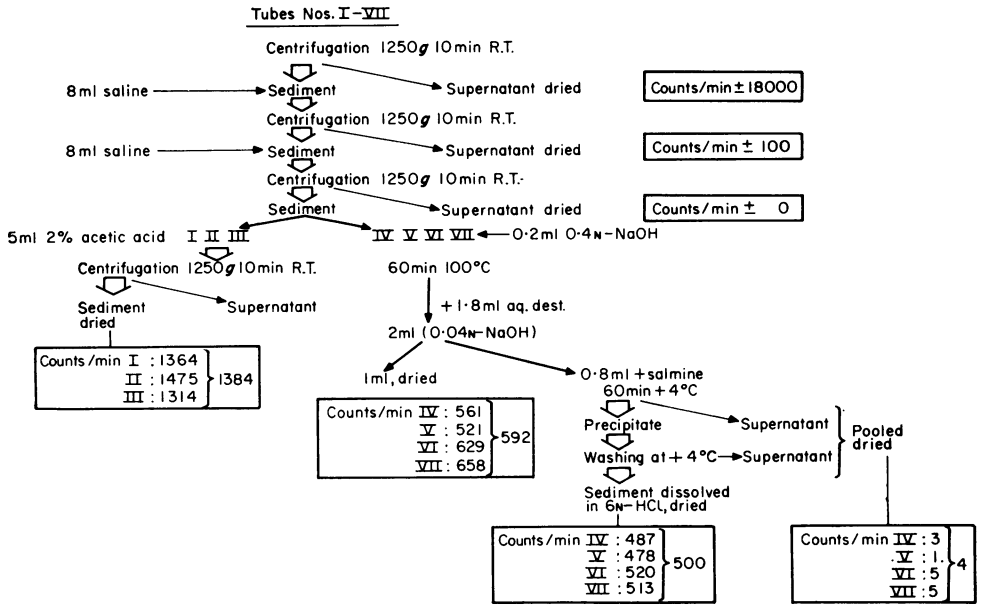

FIG. 1. Protocol of the experiments to evaluate the correlation between the total radioactivity incorporated by stimulated lymphocytes and the amount recovered from the DNA.

The correlation between total thymidine concentration in the medium and its incorporation by PHA-stimulated lymphocytes

Four parallel experiments were performed. Each experiment used the blood of a different donor. Every experiment consisted of four groups of triplicate tubes. After 48 hr of

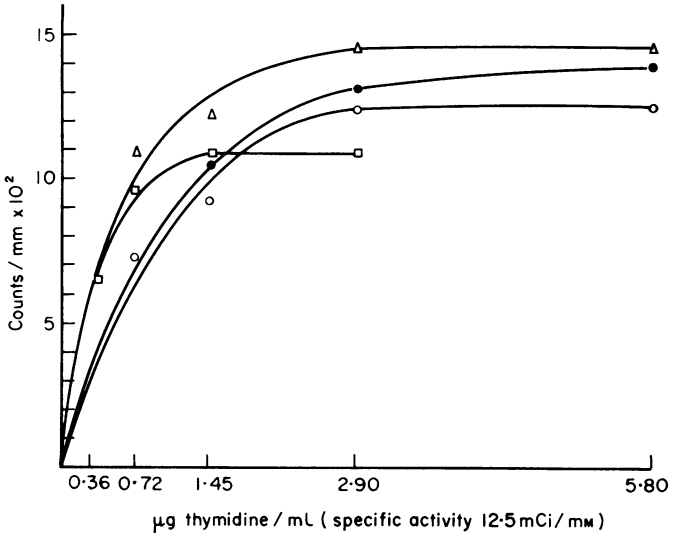

FIG. 2. The different symbols (△ ● ○ □) represent experiments with different blood samples. For technical details see text.

incubation with PHA, thymidine-2-^{14}C of a specific activity of 12·5 mCi/mM was added in a different final concentration. The incorporation was determined 24 hr later.

Fig. 2 shows the results. As can be seen the thymidine concentration in the medium has, below a certain level, a definite effect upon the incorporation. If the thymidine concentration is above 2·9 μg/ml such an effect is hardly demonstrable. Because of these results it was decided to add routinely to every culture tube 0·1 thymidine-2-^{14}C if a specific activity of 12·5 mCi/mM, resulting in a total activity of 0·6 μCi and a final thymidine concentration in the medium of 2·9 μg/ml.

Thymidine incorporated on different days of culture

Five experiments were performed. In three experiments thymidine-2-^{14}C incorporated by PHA-stimulated lymphocytes was determined after the first, second, third and fourth 24 hr, in the two other experiments these studies were extended to the 6th and 7th day. Fig. 3

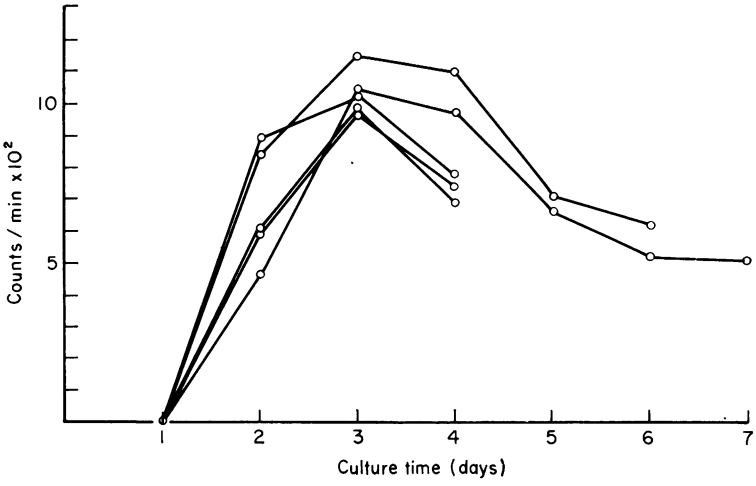

FIG. 3. Thymidine incorporation on the different days of culture. For technical details see text. The lines are connecting points from triplicate cultures.

shows the results. In all experiments a curve with the same characteristics could be obtained: The DNA synthesis starts between 24 and 48 hr of incubation, reaches a maximum between 48 and 72 hr and decreases afterwards. From these results, which were entirely consistent with the findings of others (Nowell, 1960; Börjeson, Gardell & Norden, 1966a; Hartog, Cline & Grodsky, 1967) it was decided that to measure the reactivity of PHA-stimulated lymphocytes, the DNA synthesis should be determined between 48 and 72 hr of incubation.

Influence of duration of incubation with thymidine-2-^{14}C upon the incorporation

Six parallel experiments were performed, the results of which were consistent to each other. One such experiment will be described in detail: In each experiment fifteen tubes with PHA-stimulated lymphocytes of one donor were used. To nine tubes thymidine-2-^{14}C was added after 48 hr of incubation and the incorporation determined in triplicate cultures after 56, 64 and 72 hr. To the other six tubes thymidine was added at 56 and 64 hr and the incorporation determined in triplicate at 72 hr. The results of one such experiment are

shown in Fig. 4. It can be concluded from these experiments that the incorporation of thymidine-2-^{14}C under these circumstances is linear during at least these 24 hr. It was decided to use an incubation period of 24 hr because in this way high incorporation could be achieved with relatively small amounts of radioactivity.

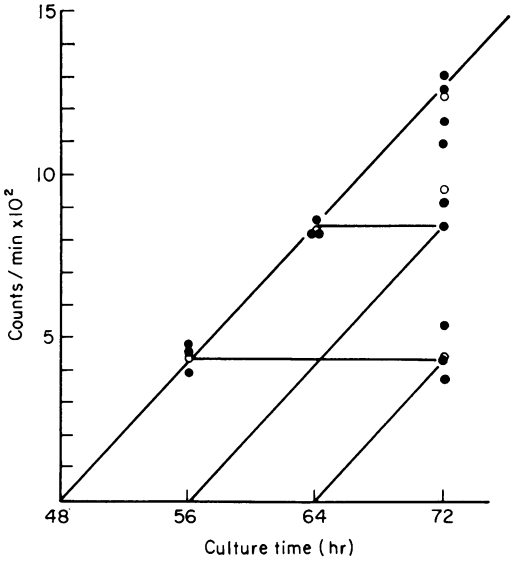

FIG. 4. Correlation between incorporation and time of incubation with thymidine-2-^{14}C. The open circles are the mean values of triplicate determinations, each of which is represented by a solid circle. A line is drawn through the values that were obtained after 8, 16 and 24 hr of incubation with thymidine, added at 48 hr. Parallel lines were drawn beginning at 56 and 64 hr and terminating at 72 hr. These points were then connected parallel to the abscissa and to the line representing thymidine incorporation beginning at 48 hr.

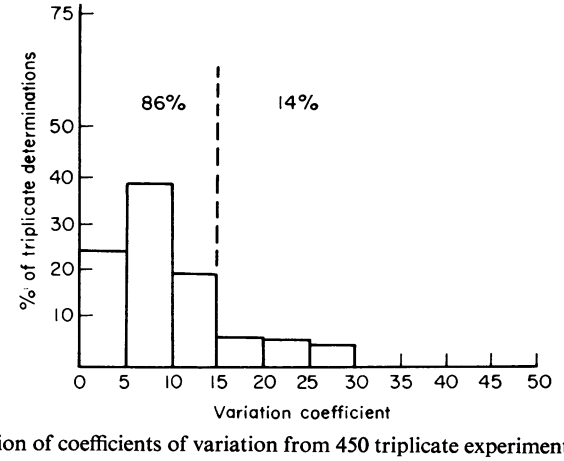

FIG. 5. Distribution of coefficients of variation from 450 triplicate experiments (1350 cultures).

The reproducibility of the measurements of thymidine-2-^{14}C incorporation and the reproducibility of the tissue culture experiments as such

Four hundred and fifty experiments were performed in triplicate giving a total of 1350

cultures. The thymidine incorporation was determined for each triplicate experiment, and the coefficient of variation was calculated. Fig. 5 shows the distribution of these 450 coefficients. Eighty-four per cent of all triplicate experiments have a coefficient of variation less than 15%. To evaluate the source of this variation the following experiment was performed: lymphocyte cultures of 12 ml with the normal cell concentration were made, thymidine was added at the usual concentration, but before harvesting the culture was divided into three equal parts and each part was handled separately to determine DNA synthesis. Between three such samples the coefficient of variation is always less than 2.

TABLE 1. Comparison of different media and different buffer systems on DNA synthesis of PHA-stimulated cells

Medium	Buffer	Incorporation (counts/min)
MEM-S	BIC.	1009
MEM-S	Tris	1347
199	BIC.	1167
199	Tris	1054

TABLE 2. The influence of serum from different sources

Donor	Serum	Counts/min	
		With PHA (3rd day)	Without PHA (6th day)
I	AB	1343	2
	FCS	1197	142
II	FCS	1248	488
III	FCS	1193	150
IV	FCS	1043	48
V	AB	1224	5
	Autologous	1223	3
VI	AB	1152	11
	Autologous	911	2
VII	AB	1145	14
	Autologous	1098	6

The influence of different media and different buffer systems on thymidine-2-^{14}C incorporation of PHA-stimulated cells

The media TC 199 and MEM-S were both tested with either bicarbonate or Tris–HCl as a buffer system. The comparison was made using the lymphocytes of the same donor on the same day. The results are given in Table 1. No significant differences between the tested media were demonstrated.

The influence of different serum sources and concentrations

The three different kinds of serum were tested both with PHA (3rd day) and without (6th day). The results are given in Table 2. No significant differences in DNA synthesis

after PHA stimulation were found. However, the control values on the 6th day without PHA, show a definite stimulation with FCS, which is absent when autologous serum or AB serum pool is used. Five experiments were performed with different concentrations of the human AB serum pool. Every experiment was performed in triplicate with the lymphocytes of one donor. As can be seen in Table 3, 20% serum was either optimal or equal to optimal.

TABLE 3. Influence of different concentrations of human AB serum-pool upon DNA synthesis of PHA-stimulated lymphocytes

Serum concentration in the medium (%)	Counts/min				
	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V
10	86	70	1137	693	917
20	1224	1152	1605	1190	1359
30	1208	826	1443	1106	1405
40	875	858	1254	977	1336

The influence of the presence of granulocytes in the tissue culture

A comparison was made between lymphocyte cultures with less than 0·5% granulocytes and similar cultures, to which 6×10^6 pure granulocytes per tube were added, so that a lymphocyte–granulocyte ratio of 1:2 was obtained. This is about the same ratio as one gets, when no purification of the lymphocytes is carried out. The thymidine incorporation between 48 and 72 hr in both groups of triplicate cultures did not differ signifficantly.

TABLE 4. The influence of different cell concentrations upon PHA-stimulated DNA synthesis

Total No. of cells/ tube (4 ml) ($\times 10^6$)	Counts/min		
	Exp. I	Exp. II	Exp. III
1			467
1·5	862	622	
2			946
3	1605	1190	1230
4			1012
5			1004
6	2184	1084	

The influence of different cell concentrations

Three experiments were performed, in each of which triplicate cultures of 4 ml with different lymphocyte concentrations were set up. To all the tubes 0·05 ml of PHA was added. The usual amount of thymidine-2-^{14}C was added to the cell cultures with 3×10^6 lymphocytes or less per tube as well as to all the tubes of Experiment III. To the tubes with 6×10^6 lymphocytes in Experiment I and II twice the amount was added. The results are given in Table 4. It can be said that there is a linear relationship between the number of lymphocytes and the thymidine incorporation if the first does not exceeed 3×10^6/tube.

The influence of different PHA concentrations

Five experiments were performed, each with the lymphocytes of one donor. Table 5 gives the results. It can be seen that under these circumstances 0·05 ml of this PHA preparation is either optimal or equal to optimal.

TABLE 5. The influence of different PHA concentrations

PHA added per tube (ml)	Counts/min				
	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V
0·025		1268	1116	1139	1012
0·05	1489	1605	1178	1186	1065
0·1	1224	1317	896	1022	961
0·2	474	655			
0·4	53				

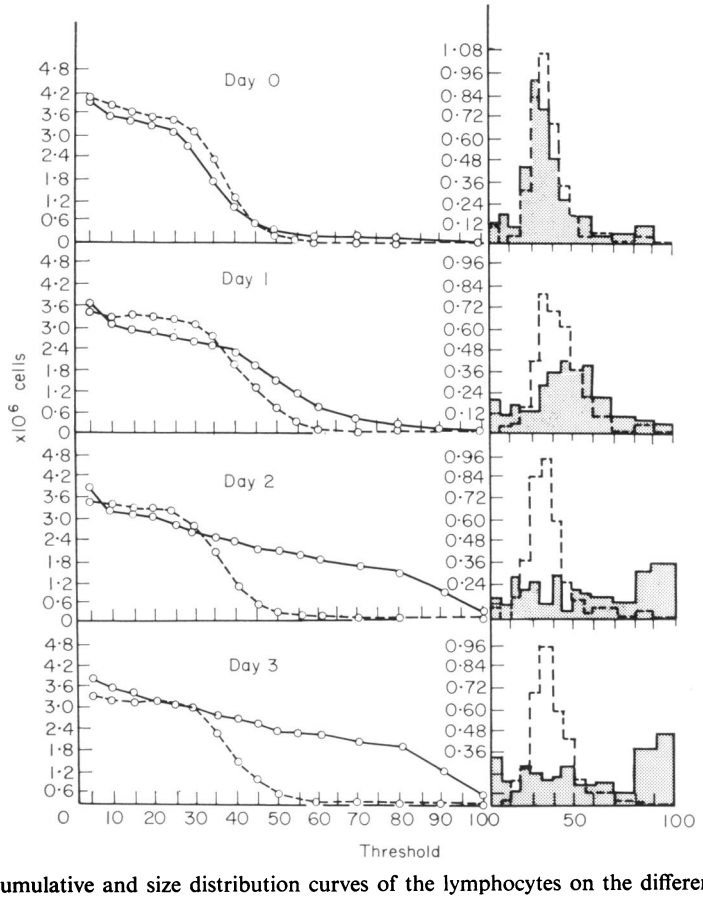

FIG. 6. Cumulative and size distribution curves of the lymphocytes on the different days of culture.

The number of cells present on different days of culture, their size distribution and their total DNA content

At zero time and after 24, 48 and 72 hr of incubation two tubes with and two tubes without PHA were treated for cell counting as described. Fig. 6 shows the curves obtained

TABLE 6. The total number of cells in culture present on the different days with and without PHA stimulation compared with the total DNA content

Hours of incubation	With (+) or without PHA (−)	Total No. of cells present ($\times 10^6$)	Total DNA content (mg)
0	+	3·5	33·9
24	+	2·9	25·5
48	+	3·0	25·5
72	+	3·1	32·0
0	−	3·6	35·4
24	−	3·3	—
48	−	3·2	28·9
72	−	3·1	23·7 (?)

at increasing threshold settings and the distribution curves as they can be calculated for the different days. It can be seen from the curves that the total number of cells can be read at threshold 20. In the same experiment the total DNA content of the cultures on the different days was determined. The total cell counts and the total DNA contents are given in Table 6.

DISCUSSION

Lymphocyte transformation *in vitro* has been the subject of a great number of studies, many of which needed a quantitative or semi-quantitative evaluation of the reactivity. Although it is claimed that with morphological interpretation of the blastogenesis reproducible results can be obtained by very experienced and skilful investigators, we prefer a method that is objective and the reproducibility of which is easily determined.

The measurement of DNA synthesis of lymphocytes *in vitro* by thymidine incorporation has been described by several authors (Dutton & Pearce, 1962; Michalowski, 1963; Bain *et al.*, 1964; Aisenberg, 1965; Caron *et al.*, 1965; Sell & Gell, 1965; Börjeson *et al.*, 1966b; Hartog *et al.*, 1967); but all these techniques show major and minor differences and very often it is not quite clear why a particular method was followed. Moreover, data about the reproducibility are scarce.

In our experiments we chose the lymphocyte culture conditions, described by Hirschhorn *et al.* (1963) and modified these when indicated. The DNA synthesis determination was based upon the one described by Jasinska & Michalowski (1962). First it could be demonstrated that under our experimental conditions only a negligible amount of radioactive thymidine not present in DNA was demonstrable, which indicates that thymidine incorporation under our conditions reflected DNA synthesis. With regard to the investigations concerning the thymidine concentration and the different periods of incubation the following can be remarked: Milton, Cooper & Halle-Panneko (1965) showed that thymidine in tissue culture can be degraded to thymine and dihydro-thymine, which are not incorporated into the DNA.

When Caron *et al.* (1965) described their method to measure lymphocyte transformation by thymidine incorporation Cooper (1966) argued that because of the degradation only short periods of incubation with radioactively labelled thymidine could be used for reliable DNA synthesis determination.

Our results, however, show that if the thymidine concentration is chosen well above the range where the incorporation is concentration-dependent the uptake of labelled thymidine is linear during 48–72 hr. On theoretical grounds a longer period of incubation is preferable since more cells in this asynchronic culture will enter the S period. Comparison of the experimental conditions shows that the amount of thymidine per lymphocyte used by Cooper, Caron and us is in the ratio of 1:25:120, not even considering the fact that Caron did not remove the polymorphonuclear leucocytes.

The determination of the DNA synthesis seems to be more reproducible than the synthesis in replicate culture tubes from the same individual. It was decided to perform every experiment in triplicate and determine the coefficient of variation. If the coefficient was more than 15% no significance was given to the results.

From the comparison between the two media it may be concluded that both are suitable. A Tris-buffer was compared with the original bicarbonate buffer because the latter may necessitate pH-corrections or a CO_2 incubator. The results show that Tris-buffered media are in no way inferior.

When the DNA synthesis of PHA-stimulated lymphocytes in media supplemented with sera from different sources was compared, it became clear that all these kinds of sera allowed a good reactivity. However, the reactivity measured in the negative control tubes on the 6th day showed striking differences. As has been described by others (Johnson & Russell, 1965), the lymphocytes of some individuals show definite stimulation if cultured in the presence of FCS without PHA. This has been called spontaneous transformation (Sabesin, 1965). The fact that the same lymphocytes in pooled human AB serum do not show such a reactivity indicates that instead of spontaneous transformation, stimulation of some kind is more likely, e.g. by heterologous serum proteins. Since it has been described by many others that there are differences in the way some human sera allow lymphocyte reactivity in tissue culture, we decided to use large human AB serum pools instead of autologous serum. Such a pool lasted for several months and allowed a good comparison between the results. Initially some difficulties were met when old samples of the serum pool were used. A decrease of thymidine incorporation was noticed after PHA-stimulation and morphologically many dead cells were seen. It was possible to remove this inhibiting or toxic factor from the serum by filtration through a Millipore filter (0·22 μ pore size).

From Table 3 it can be concluded that an optimal nutritional environment requires 20–30% serum in the medium. The finding that the addition of polymorphonuclear leucocytes to the lymphocyte cultures did not influence the PHA mediated DNA synthesis, did not rule out the possibility that antigen stimulated and mixed lymphocyte cultures can be influenced in this way for which there may be some evidence (Jones, 1966). For this reason and to eliminate any variable it was decided always to purify the lymphocytes by leukopak filtration.

The linear relationship that was demonstrable between the cell concentration and the incorporated thymidine implies that to obtain reproducible and comparable results, accurate cell counting and differentiation in the preparation of the lymphocyte suspension is a necessity. As the conventional method with the haemocytometer produces a considerable error, the required precision could only be reached by electronic cell counting. For the same

reason special attention should be taken to use carefully adjusted volumes of the lymphocyte suspension in culture.

The experiments using different PHA concentrations in the medium show that doses higher than 0·1 ml of the standard PHA solution/4 ml resulted in a lower DNA synthesis. For this reason 0·05 ml PHA/culture tube was chosen as the standard dose.

Many aspects of the kinetics of PHA-stimulated lymphocyte cultures are still unknown and one of the most informative experiments would be the total number of cells present at any particular time. Unfortunately, until recently the clumping of the lymphocytes caused by the PHA, has interfered with such a determination. The red cell stromatolysing properties of cetrimide have been described but Cetavlon treatment at low pH resulted in a breakage of the lymphocyte clumps in PHA cultures without lymphocyte lysis (Stewart & Ingram, 1967). Microscopic observations revealed complete lysis of the red cells and a suspension of single white cells with intact cell membranes and cytoplasm. This procedure therefore allows both accurate electronic cell counting as well as determination of the size distribution through the whole culture period. The results obtained so far indicate a greater cell loss after 24 and 48 hr in PHA-stimulated cultures as compared with negative cultures. Furthermore the size distribution show a clearcut shift to larger cells during DNA synthesis. A rough calculation from these data of the number of enlarged cells at 72 hr results in 62%. The discrepancy between this value and the percentage of transformed cells as determined by several authors by morphological criteria ($\pm 80\%$, Hirschhorn, 1963), can be explained by the fact that in the microscopical evaluation small cells with altered nuclear structures are interpreted to be transformed. Comparison of the total number of cells present on the different days with the total DNA content shows that the latter parallels the drop at 24 and 48 hr in the PHA-stimulated cultures. However, at 72 hr the DNA content is increased again, and much more than the number of cells. This could be explained by the presence of cells in the late S or G_2 phase.

It is our opinion that significance should only be given to results demonstrating major changes. Many more of these experiments will have to be performed, but we think that the application of the cell counting technique combined with the determination of both the total DNA content and the DNA synthesis offers a way to further study of the kinetics of lymphocyte cultures.

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