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STANDARDIZATION OF LYMPHOCYTE TRANSFORMATION TO PHYTOHAEMAGGLUTININ

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SUMMARY

In contrast to the million cells used by many current workers, careful analysis of lymphocyte transformation to phytohaemagglutinin determined optimum conditions as (i) 200,000 lymphocytes in 1 ml of culture fluid in 100 × 12-mm round-bottomed glass tubes (ii) phytohaemagglutinin at 1 μg/tube (iii) ^{3}H-thymidine at 1 μCi/tube (iv) with a convenient incorporation time 4 hr (v) starting 44 hr after adding phytohaemagglutinin. It was then found 10% autologous serum was the optimum concentration in the culture fluid. The log–normal range of transformation indices for young adults was 27–93–255.

INTRODUCTION

The peripheral lymphocytes of many species including human beings are activated to undergo blast formation by specific and non-specific stimulants (Knight, Ling, Sell & Oxfiard, 1965; Chalmers, Cooper, Evans & Topping, 1967; Hartog, Cline & Grodsky, 1967; Schellekens & Eijsvoogel, 1968; Sample & Chretien, 1971). This stimulation can be detected by the incorporation of ^{3}H-thymidine into DNA. Phytohaemagglutinin (PHA) activates a high proportion of the lymphocytes of most individuals, but current methods show a wide variation in the incorporation of ^{3}H-thymidine, so that the lower limit of normal is very low. Believing that much of this variation is methodological and in an attempt to tighten-up the normal range, lymphocytes from eight normal subjects have been titrated throughout six variables, (i) the number of lymphocytes, (ii) the concentration of PHA, (iii) the concentration of ^{3}H-thymidine, (iv) the incorporation time, (v) the time after addingPHA at which to begin ^{3}H-thymidine incorporation, and (vi) the concentration of autologous serum in the culture fluid.

MATERIALS AND METHODS

*Burroughs Wellcome TC*199 supplemented with 200 units/ml of penicillin and 100 μg/ml of streptomycin was used throughout the cultures.

Highly purified phytohaemagglutinin (*PHA*) was obtained from the Wellcome Research Laboratories.

^{3}H-thymidine (specific activity 28 Ci/mmol) was obtained from the Radiochemical Centre, Amersham.

Scintillation fluid NE 233 was purchased from Nuclear Enterprises Ltd and samples were counted in a Tracerlab scintillation counter.

Human blood lymphocytes were obtained by a modification of the method of Coulson & Chalmers (1964). Human venous blood was defibrinated by glass beads (2 mm diameter, 1 glass bead/1 ml of blood). The defibrinated blood was centrifuged, serum was removed and kept for further use. The remaining blood was diluted with the same volume of TC199, mixed with one-third volume of 3% gelatin in saline, which was freshly made and sterilized by ultrafiltration. The mixture was kept for 30–45 min at 37°C to sediment the erythrocytes. The lymphocyte-rich supernatant was removed, washed twice with TC199, and the lympocytes were counted.

Lymphocyte culture

Lymphocyte cultures were set up in round-bottomed glass test tubes (100 × 12 mm) with loosely fitting aluminium caps. The test tubes contained lymphocytes suspended in 1 ml of TC199 enriched 20% with autologous serum. All experiments were set up in triplicate. The cultures were placed in a desiccator jar (without desiccant), gassed with 5% CO_2 and 95% air and kept at 37°C. After 48-hr incubation ^{3}H-thymidine was added and 4 hr later the cells were harvested. The cells were centrifuged, washed twice with 2 ml of phosphate buffered saline (PBS, pH 6·8), finally resuspended in 2 ml of PBS, and 2 ml of 10% cold trichloroacetic acid were added to precipitate the nucleic acids and proteins. The precipitates were collected by centrifugation, washed with 2 ml absolute methanol to dissolve proteins, such as haemoglobin, and finally resuspended in 1 ml absolute methanol, to which 9 ml of scintillation fluid NE 233 was added. Samples were counted. Results are reported as counts per minute (cpm) or the transformation index (TI) which is expressed as:

$$TI = \frac{\text{cpm of stimulated cells}}{\text{cpm of unstimulated cells.}}$$

In experiments (i) the number of lymphocytes varied from $0{\cdot}05 \times 10^6$ to 2×10^6 per culture. The cultures contained 1 μg PHA and 1 μCi ^{3}H-thymidine and the incorporation of ^{3}H-thymidine into DNA was measured after 4-hr incorporation time.

In experiments (ii) the concentration of PHA varied from 0·01 μg to 100 μg using $0{\cdot}2 \times 10^6$ lymphocytes and 1 μCi ^{3}H-thymidine per culture.

In experiments (iii) the ^{3}H-thymidine concentration was increased from 0·01 μCi to 100 μCi and the incorporation by $0{\cdot}2 \times 10^6$ lymphocytes was measured in response to 1 μg PHA.

In experiments (iv) the incorporation time was studied by harvesting cultures at 4-hr intervals from 4 to 24 hr. Cultures contained $0{\cdot}2 \times 10^6$ lymphocytes and 1 μg of PHA, and 1 μCi of ^{3}H-thymidine was added after 48-hr incubation.

In experiments (v) sets of cultures as in (iv) of a given subject were started and ^{3}H-thymidine was added at intervals ranging from 24 to 120 hr after the addition of the PHA.

In experiments (vi) when the optimum conditions for (i)–(v) were found, the concentration of autologous serum was then varied from 0–100% in the culture fluid.

Finally normal ranges were determined for PHA response using 1,000,000 or 200,000

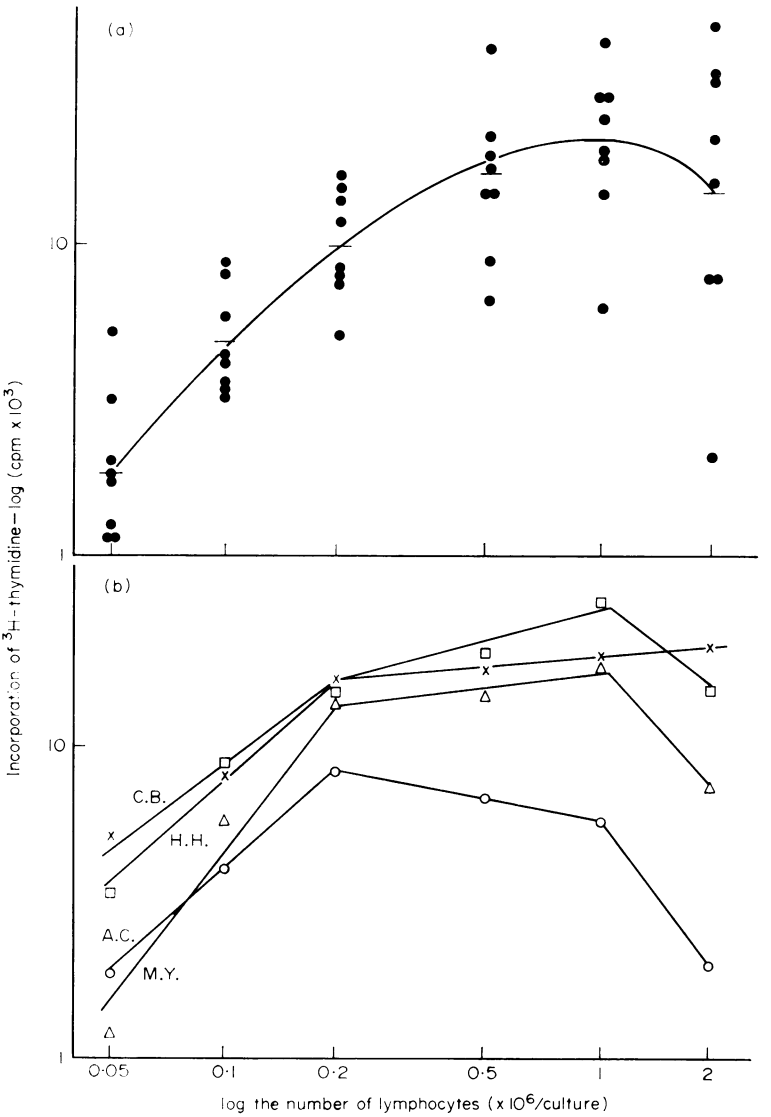

FIG. 1. The logarithm of the cpm of ^{3}H-thymidine incorporated plotted against the logarithm of the number of lymphocytes. It can be seen in (a) which incorporates all the results for all eight subjects, that the calculated mean line has lost linearity by the time there are 1,000,000 cells in the culture. (b) Shows the four of the eight subjects in whom linearity was lost after 200,000 cells.

lymphocytes. As normal subjects healthy persons from 4 to 45 years of age were used and included seventeen males and thirteen females.

RESULTS

(i) *The relationship between the incorporation of ^{3}H-thymidine and a varying number of lymphocytes* (Fig. 1)

In all eight normal subjects a linear increase of incorporation was observed from $0{\cdot}05 \times 10^6$ up to $0{\cdot}2 \times 10^6$ lymphocytes per culture (Fig. 1a) but four out of eight subjects failed to show a linear increase of incorporation up to $0{\cdot}5 \times 10^6$ lymphocytes (Fig. 1b). Only one really showed a linear incorporation up to 1×10^6 lymphocytes per culture. It is therefore concluded that $0{\cdot}2 \times 10^6$ lymphocytes per culture should be used to ensure the linear relationship to the incorporation of ^{3}H-thymidine in response to PHA in most individuals.

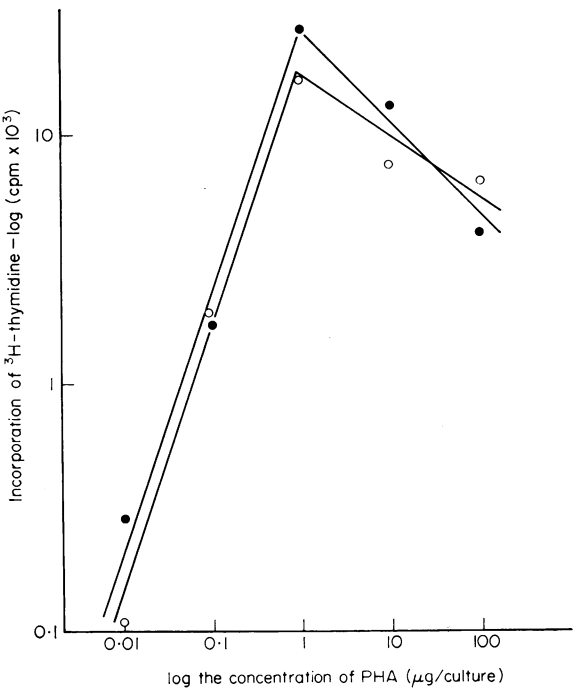

FIG. 2. The logarithm of the cpm of ^{3}H-thymidine incorporation plotted against the logarithm of the concentration of phytohaemagglutinin. In all eight normal subjects (though only two are shown) there is a linear relationship up to 1 μg/ml culture fluid.

(ii) *The PHA dose-response* (Fig. 2)

For all eight subjects there was a linear increase of incorporation of ^{3}H-thymidine related to PHA dose from 0·01 μg to 1 μg by $0{\cdot}2 \times 10^6$ lymphocytes. The decrease of incorporation above 10 μg of PHA is probably due to the cytotoxicity of PHA. It is therefore concluded that for this preparation of PHA the optimal concentration in the culture is 1 μg/ml.

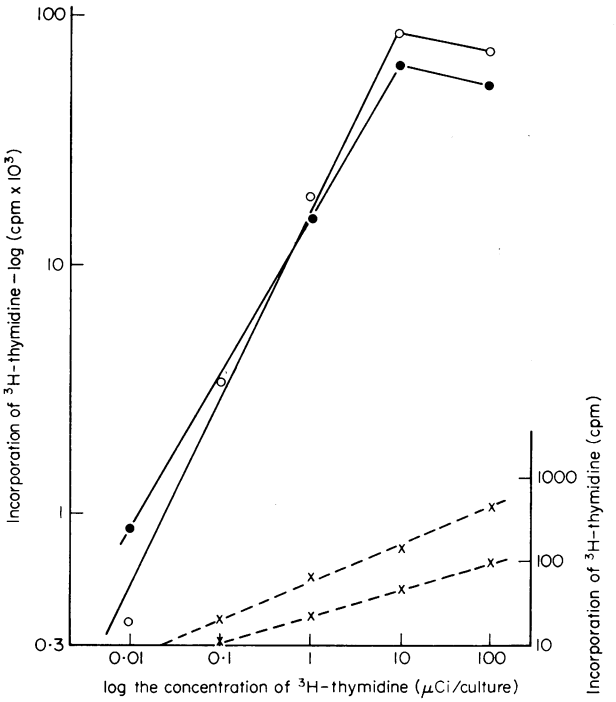

FIG. 3. The logarithm of the cpm of ^{3}H-thymidine incorporation plotted against the logarithm of the concentration of ^{3}H-thymidine in PHA-stimulated (○—○, ●—●) and unstimulated (×—×) cultures. For tests of PHA-responsiveness there is linearity up to 10 μCi/l ml culture fluid.

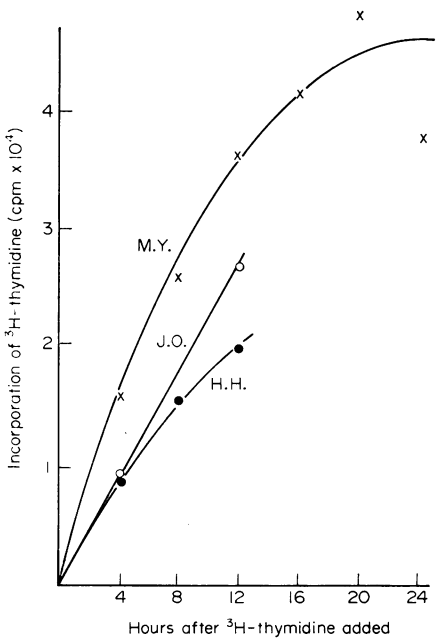

FIG. 4. The cpm of ^{3}H-thymidine incorporation plotted against the hours after the addition of ^{3}H-thymidine. A linear relationship holds for only 8–12 hr.

(iii) *The relationship between the incorporation of ^{3}H-thymidine and the rise of ^{3}H-thymidine concentration* (Fig. 3)

With the rise of ^{3}H-thymidine concentration, the incorporation increased in linear fashion up to 10 μCi. However, there was a decrease in incorporation at 100 μCi of ^{3}H-

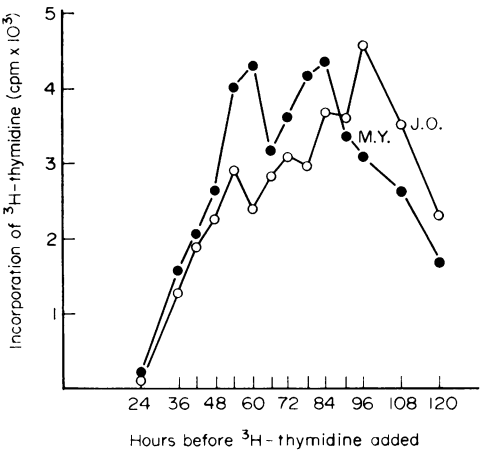

FIG. 5. The choice of when to add ^{3}H-thymidine. The cpm of ^{3}H-thymidine incorporation is plotted against the hours after PHA was added, prior to the labelling with ^{3}H-thymidine. In the two subjects shown, maximum activation to ^{3}H-thymidine incorporation is occurring 48–96 hr after PHA was added. However, this activation is only linear up to 54 hr, whereafter there is no further advantage in waiting to add ^{3}H-thymidine.

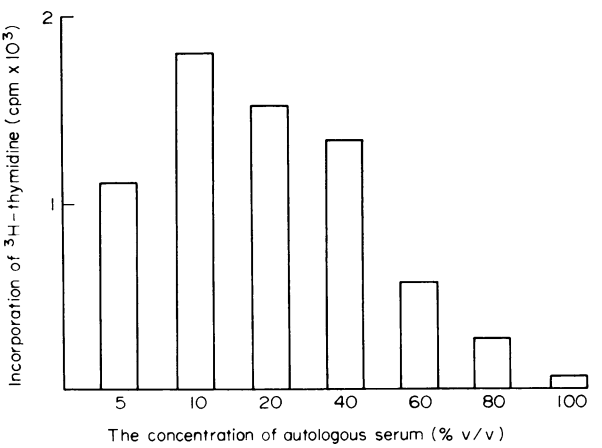

FIG. 6. The cpm of ^{3}H-thymidine incorporated plotted against the concentration of autologous serum (% v/v) in the culture fluid. In the results shown for the normal subject (confirmed in three others) the optimum concentration is clearly 10%.

thymidine which occurred in stimulated cultures even though there was a steady increased incorporation in unstimulated cultures. This decrease is presumably due to cell death caused by the irradiation by tritium. In view of the cost of ^{3}H-thymidine 1 μCi/culture was chosen as it is clearly on the linear portion of the response and yields adequate cpm.

(iv) *The incorporation time for* ^{3}*H-thymidine* (Fig. 4)

$0{\cdot}2 \times 10^6$ lymphocytes, 1 μg PHA and 1 μCi ^{3}H-thymidine being decided, the time course was studied. A linear incorporation of ^{3}H-thymidine was seen up to 12 hr in three individuals. Four hours yielded adequate cpm and was clearly on the sharply linear part of the curve.

(v) *The time after the addition of PHA at which to add* ^{3}*H-thymidine* (Fig. 5)

It can be seen that a linear increase in cpm occurs up to 54 hr after the addition of PHA. There is then no advantage till 96 hr, whereafter there is indeed a decline. As 44 hr is well up on the linear response, and is also a convenient time to enable a further 4 hr for ^{3}H-thymidine incorporation which can then be terminated during the working day, it was chosen as the optimum.

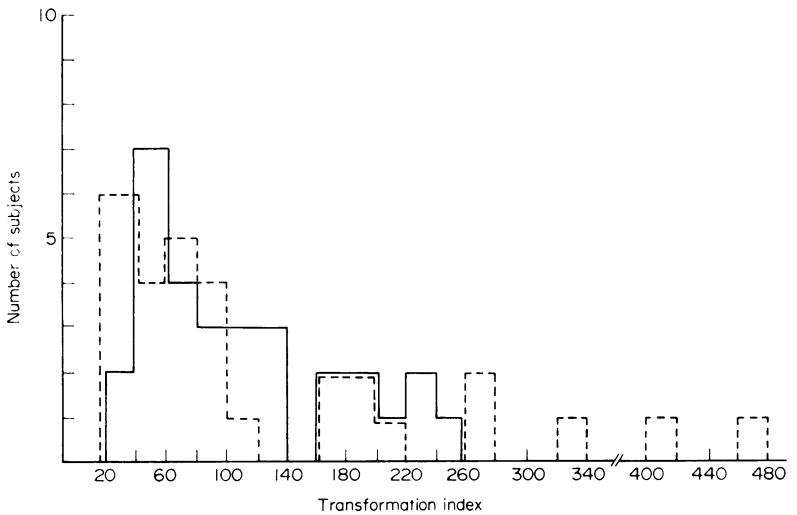

FIG. 7. Frequency distributions of transformation indices using either 200,000, (———) or 1,000,000 (– – – –) lymphocytes per culture. There is an apparently log-normal distribution confirmed by the calculated log-normal ranges closely fitting the observed (see Tables 1 and 2). Furthermore it is clear that the use of 200,000 cells yields less scatter and a somewhat tighter log-normal range than does the use of 1,000,000 cells.

(vi) *The concentration of autologous serum*

Fig. 6 shows the results for a given subject using different concentrations of autologous serum in TC199. Almost identical results were obtained in three other normal subjects so that 10% autologous serum was the optimum concentration. With no added serum no incorporation of thymidine occurred.

Fig. 7 shows the log-normal distributions found using both 1,000,000 or 200,000 lymphocytes in each tube. Tables 1 and 2 show the actual cpm achieved and it is clear that the transformation indices at 200,000 cells are less scattered than when using 1,000,000 cells. There was no obvious difference at either end of the ages studied (4–45 years) nor any significant statistical difference between the sexes.

TABLE 1. PHA response in normal subjects (1×10^6 lymphocytes)

Normal subjects	Cpm		TI
	Unstimulated	PHA stimulated	
1	1,262	18,565	15
2	250	6,818	26
3	627	17,153	27
4	280	7,520	28
5	118	4,020	34
6	1,037	38,719	37
7	517	21,131	41
8	161	7,012	44
9	523	30,516	58
10	668	38,724	58
11	82	5,023	61
12	120	8,403	70
13	444	31,038	70
14	580	42,243	73
15	68	5,128	74
16	326	27,246	83
17	72	6,119	85
18	214	17,815	85
19	132	12,331	94
20	354	37,853	107
21	222	36,341	164
22	91	16,625	179
23	290	57,249	187
24	446	88,116	198
25	222	45,154	204
26	505	131,299	260
27	91	24,689	271
28	128	41,872	327
29	43	17,860	415
30	35	16,058	460
Median	225	19,912	88
+2 SD	1,433	117,470	512
−2 SD	35	3,375	15

SD = standard deviation.

TABLE 2. PHA response in normal subjects ($0{\cdot}2 \times 10^6$ lymphocytes)

Normal subjects	Cpm		TI
	Unstimulated	PHA stimulated	
1	98	2,275	23
2	78	2,432	31
3	39	1,552	40
4	49	2,466	50
5	160	8,544	54
6	30	1,659	55
7	104	5,814	56
8	44	2,546	58
9	138	7,990	58
10	72	5,150	72
11	122	9,259	76
12	29	2,262	78
13	68	5,364	79
14	91	7,677	85
15	19	1,684	89
16	32	2,973	93
17	66	6,554	100
18	50	5,606	112
19	24	2,786	116
20	98	12,071	123
21	35	4,380	125
22	108	14,236	132
23	46	5,757	160
24	38	6,651	175
25	57	10,348	181
26	83	15,053	181
27	38	8,072	212
28	84	19,817	236
29	72	16,395	238
30	49	11,993	245
Median	59	5,690	93
+2 SD	174	24,144	255
−2 SD	20	1,223	27

SD = standard deviation.

DISCUSSION

More than 1,000,000 lymphocytes have been used for the assessment of transformation by measuring the incorporation into DNA of tritiated thymidine (Michalowski, 1963; Caron, Sarkany, Williams, Todd & Gell, 1965; Wilson & Thomson, 1968; Simons, Fowler & Fitzgerald, 1969; Fitzgerald, 1971; Sample & Chretien, 1971). It was not fully established how many lymphocytes ought to be used in those assays.

Here it has been shown that $0{\cdot}2 \times 10^6$ lymphocytes can, in fact, be better than 1×10^6 cells per culture in yielding a tighter normal range of transformation indices (see Table 1, 2 and Fig. 7). Furthermore, this seems to be the first clear demonstration of the log–normal distribution of such indices. In that the calculated log–normal ranges closely fit the observed (see Tables 1 and 2) they would also seem to be the first correct 95% reliable normal ranges recorded.

It is important for clinical use to reduce the number of lymphocytes and the concentration of serum, as many blood samples have to be taken in infancy and childhood. The effects of serum on transformation have to be considered. Autologous serum was used throughout this study because: (i) foetal calf serum can contain factors inhibiting the lymphocyte transformation by up to 90% in comparison with autologous serum; (ii) of difficulties in obtaining fresh AB serum; (iii) although the use of serum-free media has been reported (Coulson & Chalmers, 1967; Mattsson & Lindahl-Kiessling, 1971) the problem in such assays is the large number of lymphocytes (more than 2×10^6/culture) required for the expression of transformation.

In clinical conditions serum factors which block or enhance are encountered (Havemann, Dosch & Burger, 1970) and it is therefore important to test autologous serum. In normal subjects it seems likely that the optimum expression of transformation depends on a balance between such factors. Among many cancer patients with blocking factors at 20% autologous serum, Dr Caroline Butterworth and I (to be published) have found that when 10% autologous serum is used the inhibition is often lost. When it becomes possible to count T- and B-lymphocytes by simple methods, it should be possible to adjust random blood samples to a fixed number of T-lymphocytes and thereby produce even tighter and clinically more valuable normal ranges for transformations, not only to PHA, but also to a standard candida immunogen, and perhaps even a standard mixed-lymphocyte immunogen. For the latter two tests it seems again the above parameters have not been adequately examined.

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