The influence of incubation temperature on the rate of human lymphocyte proliferation in vitro

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Summary. Using the FPG staining technique we have confirmed that incubation temperature has a marked effect on the proliferation rate of cultured lymphocytes. With 48 h-cultures the proportion of 2nd-cycle cells increases linearly over the temperature range 36–39 °C. However the absolute size of the increase exhibits interdonor variability.

With the introduction of the fluorochrome-plus-Giemsa (FPG) technique[2] for staining chromosomes to produce the 'harlequin' effect, it has become possible to determine simply and reliably whether a metaphase is in its 1st, 2nd or subsequent in vitro cell cycle. This is valuable for example in enabling the analysis of chromosomes for unstable aberrations to be restricted to guaranteed 1st division cells. For many years the principal means of limiting the number of 2nd division cells analyzed was to harvest the cultures at about 48 h, but using the FPG technique it was demonstrated that appreciable numbers of 2nd division cells may be present even at this time[3]. Quite large variations in lymphocyte proliferation rates were also observed amongst laboratories and even between donors within one laboratory[4–6].

One factor which could account for the variability is the temperature at which the lymphocytes are incubated. This was briefly investigated in 1969 by Bender and Brewen[7] who observed a marked effect on mitotic indices at several incubation times and temperatures, and more recently by Borodkin[8] who reported an earlier start to mitotic activity in both irradiated and control cultures which were incubated at 39 °C rather than 37 °C. Salodino and Johnson[9], working with hamster fibroblasts, consider that the length of S phase is reduced at higher temperatures as there is a faster synthesis of DNA.

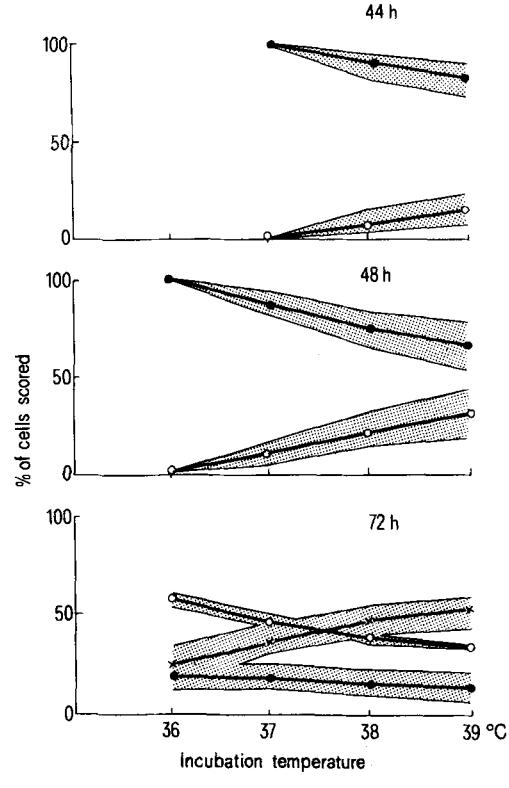
An efficient water-jacketed incubator should regulate the internal temperature closely with negligible gradients within the cabinet. The stability however is disturbed by opening the door and by introducing cultures at room temperature. A batch of cultures inserted without prewarming can take 1–2 h to reach 37 °C depending on the number incubated, and their introduction can also cause the temperature of cultures already in the incubator to drop by 1–2 °C for a similar period (unpublished observations). Together these factors could result in a considerable shortening of the effective incubation time, thereby probably slowing the rate of progress of cells through the cycle. This may unwittingly serve to reduce the contamination of the cultures with 2nd division cells. Transient temperature fluctuations are reduced with water baths because of the more rapid heat transfer, but it is easy to show that the temperature of liquid in partially submerged culture vessels e.g. Lowenstein bottles may drop by 1 or 2 °C as a result of briefly removing the lid of the bath.

Evidently incubation times and conditions may be less constant than one assumes and in the present paper the FPG technique is used to examine the extent to which small variations in temperature might explain the apparent diversity of proliferation rates.

Materials and methods. Samples of venous blood were obtained from 4 healthy adults. For each donor 18 replicate microcultures were prepared with 0.3 ml heparinized whole blood, 4 ml Eagle's MEM, 0.5 ml bovine serum and 0.1 ml PHA; the BrdU concentration was 7.2 µM. Cultures were incubated in covered water baths maintained at 36, 37, 38 or 39 °C. Temperature fluctuations in the water were carefully monitored and found to be ±0.1 °C. Colcemid was present for the final 3 h of incubation and the cultures were fixed after 44, 48 or 72 h. Full details of the culture and fixation procedures have been published elsewhere[10] and the FPG staining technique was based on that described by Perry and Wolff[2]. For each donor, incubation temperature and fixation time, 200 metaphase spreads were examined on the basis of their staining reaction as being in their 1st, 2nd or later in vitro cell cycle.

Results and discussion. The table records the number of cells observed at their 1st, 2nd or subsequent in vitro metaphase for the 4 donors, 4 temperatures and 3 fixation times. Mean values have been calculated for the 4 donors and these are shown in the figure.

The results confirm that the rate of lymphocyte proliferation increases as the incubation temperature is raised. However, superimposed on this temperature effect, significant inter-donor differences in cell-cycle times are once again apparent. At 44 h few 2nd cycle cells were observed at 36 and 37 °C but the number then increased linearly with incubation temperature up to 39 °C where a maximum of 25% was observed for 1 donor (B). A linear increase in the yield of 2nd cycle metaphases was observed in the 48-h cultures over the entire range of incubation temperatures with up to 17% 2nd division cells at 37 °C. Donor B again displayed the most rapid proliferation with only 55% of the 39 °C cells being in cycle 1.



The percentage of 1st (●), 2nd (○) and 3rd (+) cycle metaphases observed in cultures incubated at 36, 37, 38 or 39 °C for 44, 48 or 72 h. Each point is the mean for the 4 donors, the stippled areas indicate the spread of the individual values.

The yields of 1st, 2nd and 3rd cycle metaphases in 44-, 48- or 72-h lymphocyte cultures incubated at 36, 37, 38 or 39 °C

Donor Age/Sex	Cell cycle	Fixation time (h)	Yields (%) at temperatures of 36°C	37°C	38°C	39°C
A	1	44	100	100	96	91
36/M	2		-	-	4	9
B	1	44	100	100	84	75
24/F	2		-	-	16	25
C	1	44	100	100	93	83
28/M	2		-	-	7	17
D	1	44	100	99.5	95	86
34/M	2		-	0.5	5	14
A	1	48	98	94.5	83.5	79
	2		2	5.5	16.5	21
B	1	48	100	83	66	54.5
	2		-	17	34	45.5
C	1	48	100	89.5	81	69.5
	2		-	10.5	19	30.5
D	1	48	99	85	75	69.5
	2		1	15	25	30.5
A	1	72	24.5	24	20.5	17.5
	2		58	46	37	33
	3		17.5	30	42.5	49.5
B	1	72	12	12	9	6
	2		54	43	38	34
	3		34	45	54	60
C	1	72	12	12	10	9
	2		60	48.5	40	33
	3		28	39.5	50	58
D	1	72	24.5	23	22	20.5
	2		60	47	39	35
	3		15.5	30	39	44.5

Presumably at some temperature the linear increase in the proliferation rate, such as that displayed by these 2nd cycle cells, would reach a peak but there is no indication from the data of the point at which this would occur. Our temperatures were chosen to reach the maximum likely to be found in a laboratory incubator set at a nominal 37 °C.

At 72 h only a minority of the cells were in 1st division and by this time 3rd and subsequent divisions accounted for a considerable fraction of the metaphases. The contribution from cycle 1 declined in case B to only 6% after incubation at 39 °C. 2nd-cycle cells also become less frequent at the higher temperatures because of earlier recruitment to cycle 3 which accounts for 45–60% of all cells at 39 °C.

It is now well accepted that the FPG method has contributed to the accuracy of scoring lymphocytes for unstable aberrations caused by mutagenic chemicals or ionising radiation. With non-FPG stained material examined for the presence of unstable chromosome aberrations, small changes in incubation temperature could distort the aberration yields and hence their interpretation in terms of, say, radiation dose. In a blood sample containing a mixture of irradiated and unirradiated cells, which is characteristic of a partial-body exposure, the faster proliferation rates at higher incubation temperatures would enhance the selective loss of those cells carrying unstable chromosome aberrations. However, the use of FPG-staining will remove this source of experimental error. Provided that similar aberration yields are obtained with 1st division cells identified by the FPG staining reaction, irrespective of how long they take to reach metaphase, highly variable proliferation rates can be ignored. However, evidence on this point is conflicting. Scott and Lyons[11], Leonard and Decat[5], conclude that 1st division cells are equally sensitive to the induction of aberrations by radiation but Bianchi et al.[12] and Wyszynska et al.[13] claim that sensitivity varies with the fixation time. Clearly some acceptable means of synchronizing the dividing cultures would be advantageous at least until uniform sensitivity of all PHA-responsive 1st division cells is established beyond doubt.

Conclusions. The present study has confirmed that incubation temperature can have a marked effect on lymphocyte proliferation in vitro and small but systematic inter-laboratory differences may well cause some of the disparities in the data published so far. However, in view of the very accurate temperature control and the uniformity of the replicate cultures in this experiment, it is clear that some other factor intrisic to the donors themselves is also involved.

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