Effect of Length of Exposure to Cell-free Medium from Mixed Leucocyte Cultures on Blastogenesis in Leucocyte Cultures from Single Subjects

MATERIAL which stimulates blastogenesis of allogeneic leucocytes is released into the surrounding medium when human leucocytes are cultured *in vitro*[1-3]. We have investigated the effect of length of exposure to cell-free medium from mixed leucocyte cultures on blastogenesis in leucocyte cultures from single donors. We also found the minimum volume of cell-free culture medium which stimulates DNA synthesis in cultures of leucocytes from single donors.

The subjects were healthy volunteers; the culture technique has already been described[4]. The leucocytes were washed once with medium 199 and were resuspended in fresh plasma from several donors. The cell suspensions were diluted with medium 199 so that the final plasma concentration was 20 per cent and the leucocyte count was 1,500/mm^3. The cell suspensions were incubated in 17 × 100 mm disposable plastic culture tubes. Cell-free culture medium was prepared from 5-day mixed leucocyte cultures as described previously[1]. In experiments investigating the relation of volume of cell-free culture medium to stimulatory activity, various volumes of the medium were added to freshly prepared leucocyte cultures from single donors. The total volume in each culture tube was 4 ml. In experiments to determine the effect of length of exposure to cell-free medium on blastogenesis in leucocyte cultures, 2 ml. of cell-free medium was added to each tube containing freshly prepared cultures of leucocytes from single subjects. After incubation for 10, 30, 60 or 120 min, three culture tubes were centrifuged at 1,500 r.p.m. for 10 min. The supernatants were decanted and the cells were resuspended in medium 199. The cells were washed twice with 4 ml. of medium 199 and were finally resuspended in medium 199 containing 20 per cent fresh pooled plasma. Control cultures, which had not been exposed to cell-free medium from 5-day mixed cultures, were also washed after incubation for 10 min and resuspended in fresh medium with plasma. At the same time, three culture tubes containing stimulatory cell-free culture medium were washed twice with medium 199 and the cells were resuspended in the original culture medium which had been removed after the first centrifugation and set aside. The cultures were incubated at 37° C for 5 days.

At the end of the incubation period, tritiated thymidine (specific activity 5·0 Ci/mmole) was added to each tube to give a concentration of 1 μCi/ml. After incubation for 2 h at 37° C, the cells were washed once in cold physiological saline, and then twice in 5 per cent trichloroacetic acid. DNA was extracted by the Schmidt–Tannhauser technique[5], except that the concentration of potassium hydroxide was decreased to 0·5 N. The radioactive content of the DNA fraction was counted in a liquid scintillation counter. Quenching was measured by an internal standardization method[6] using ^{3}H-toluene as the internal standard. Quenching was similar for the samples tested. The concentration of DNA present in each preparation was determined by measuring the optical density at 260 mμ in a spectrophotometer with a hydrogen lamp. The results were expressed as c.p.m. per 10 μg of DNA.

Table 1 shows that the stimulatory activity of cell-free culture medium was increased by increasing the volume of the medium added. Stimulation induced by 4 ml. of cell-free medium was greater than that by 2 ml. (ref. 1). There was no stimulatory activity with 0·25 ml. of cell-free culture medium. When 0·5 ml. of cell-free medium was added, DNA synthesis was slightly but consistently higher than in control cultures of unmixed leucocytes.

Table 2 shows the effect of length of exposure to cell-free culture medium on blastogenesis in cultures of leucocytes from single donors. When the stimulatory medium was removed after the first 60 min incubation, DNA synthesis was consistently higher than in unstimulated control cultures. In some cases (experiments 1 and 2 in Table 2) DNA synthesis was significantly increased with exposure for only 10 min. The effect after brief contact could not have been caused by a residue of stimulatory medium left after washing the cells. The residue after two washings was much less than 0·25 ml., which was inactive (Table 1).

Table 1. RELATION OF VOLUME OF CELL-FREE CULTURE MEDIUM TO STIMULATORY ACTIVITY

	^{3}H-thymidine uptake: c.p.m./10 μg of DNA					
	Leucocyte cultures* from single donors containing cell-free culture medium from 5-day mixed cultures					Control cultures
	Volume of cell-free medium added in each culture tube					of unmixed
Experiment No.	2 ml.	1 ml.	0·75 ml.	0·5 ml.	0·25 ml.	leucocytes
1	6,168 ± 397†	2,243 ± 146	1,995 ± 75	1,808 ± 115	1,553 ± 82	1,546 ± 35
2	4,070 ± 408	4,527 ± 201	3,525 ± 357	2,400 ± 38	1,820 ± 185	1,571 ± 163
3	3,359 ± 155	2,487 ± 109	2,421 ± 234	2,044 ± 236	1,490 ± 195	1,647 ± 93
4	6,112 ± 515	4,218 ± 209	2,671 ± 109	1·979 ± 150	1,337 ± 198	1,450 ± 137
5	6,245 ± 400	3,491 ± 133	2,268 ± 236	1,934 ± 44	1,516 ± 124	1,318 ± 171

* Number of leucocytes in each culture tube was 6 × 10^6. † Average value obtained from triplicate 5-day cultures ± *S.D.*

Table 2. EFFECT OF LENGTH OF EXPOSURE ON STIMULATORY ACTIVITY OF CELL-FREE CULTURE MEDIUM

	^{3}H-thymidine uptake: c.p.m./10 μg of DNA					
	Leucocyte cultures from single donors exposed to 2 ml. of cell-free medium from 5-day mixed cultures					Control cultures
	Length of exposure					of unmixed
Experiment No.	10 min	30 min	60 min	120 min	5 days	leucocytes
1	3,030 ± 282*	3,738 ± 205	2,621 ± 283	2,264 ± 301	7,316 ± 1,763	1,409 ± 200
2	2,062 ± 173	1,928 ± 163	1,738 ± 138	2,922 ± 233	7,359 ± 770	1,147 ± 340
3	756 ± 87	456 ± 61	1,183 ± 88	1,177 ± 71	4,813 ± 389	718 ± 127
4	1,413 ± 24	1,357 ± 137	1,778 ± 100	—	3,221 ± 83	1,199 ± 37
5	896 ± 82	1,236 ± 147	2,512 ± 138	—	7,360 ± 314	768 ± 29
6	996 ± 296	1,862 ± 83	2,747 ± 343	—	3,115 ± 219	1,241 ± 167
7	2,201 ± 264	2,165 ± 130	3,705 ± 274	—	7,368 ± 494	1,629 ± 138

* Average value obtained from triplicate 5-day cultures ± *S.D.*

It seems pertinent that an irreversible process leading to blast cell transformation developed in leucocyte cultures from single subjects after contact for 60 min, or sometimes only 10 min, with cell-free medium derived from mixed leucocyte cultures.

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