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Kinetics of 21-Trisomic Lymphocytes

I. In vitro Response of 21-Trisomic Lymphocytes to PHA

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Summary. Data presented here on the quantitative ^{3}H-thymidine incorporation into DNA, after PHA mitogenic stimulation, show that 21-trisomic lymphocytes are low-responders to PHA compared with the normal-diploid ones. Their responsiveness seems to decrease with the donor's age. Autoradiographic studies clearly demonstrate that the fraction of labeled cells at the 72nd h of incubation is significantly smaller in the 21-trisomic lmphocyte population. The comparison of labeling indexes at different times of incubation (24, 48, 72 h) also indicate, in the same population, a slower increment of the portion of DNA-synthesizing cells. Discussing these data in the light of other's observations and recent progress in the knowledge of factors and mechanisms involved in the lymphocyte response to lectin mitogenic stimulus, it is suggested that differential distribution of T- and B- and/or T-cell subpopulations and a retarded cell induction time to proliferate may be two important factors negatively influencing the responsiveness of 21-trisomic lymphocyte population.

Introduction

The knowledge of the kinetics of 21-trisomic cell has an obvious theoretical interest as well as, in perspective, possible practical implications. The analysis of cell cycle markers (Mitchinson, 1969), i.e., of discrete events that occur at a particular point in the cell cycle, may reveal specific errors or compartments where morphologic and functional modifications do occur. However, the kinetics of 21-trisomic cells is still vague. Recently a few attempts have been made to test whether cells derived from subjects affected with Down's syndrome had a modified life cycle time. Most studies have been done with fibroblast-like cells from skin secondary cultures (Mittwoch, 1967; Kaback and Bernstein, 1970; Schneider and Epstein, 1972; Segal and McCoy, 1973; Kukharenko et al., 1974; Paton

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et al., 1974; Porter and Paul, 1975). Their results were generally interpreted as suggesting an increase in the total length of the cell cycle, which, however, was differently attributed to a longer duration of G_1 or S and/or G_2 phase. Two studies, to our knowledge, have so far been conducted on 21-trisomic lymphocytes (Mellman, 1970; Dutrillaux and Fosse, 1976) with sharply contrasting results.

It is commonly recognized (Cleaver, 1967; Baserga, 1976) that the duration of the total cell cycle and of its single phases is not exactly the same for every cell type of a given organism and that a number of factors, including culture conditions, may cause differences in the cell cycle of similar cells. For our investigation we chose lymphocytes. Since their division and multiplication in vitro follow their response to an antigenic stimulus, it appeared important before studying the cell cycle to analyze some properties of this response to a few mitogens. Here we report the results obtained after stimulation with phytohemagglutinin (PHA).

Materials and Methods

Lymphocyte Cultures were established from heparinized whole blood. This simple and quick technique was preferred because previous comparative studies (Serra and Arpaia, unpublished), recently confirmed by others (Bosman et al., 1975), on the proliferative activity of lymphocytes isolated by either nylon-wool filtration or ficoll-hypaque gradient, showed that a more intense blastogenesis occurred when whole blood was used. In each culture vessel 0.5 ml of whole blood was suspended into 4.5 ml of medium RPMI 1640 (Eurobio, Paris) supplemented with 20% FCS, penicillin (50 IU/ml) and streptomycin (50 µg/ml), (penicillin-streptomycin mixture, Gibco Bio-Cult, Glasgow), and PHA (M) (Difco, USA) at various concentrations. The experiment was designed in consideration of three variables: age of subjects, dose of PHA (10, 50, 100, 400, 600 µg/ml), and time of incubation (24, 48, 72 h).

Labeling. Growing cells, at fixed intervals after the initiation of incubation (23, 47, 71 h) were pulse-labeled for 1 h with ^{3}H-thymidine (Amersham, England; specific activity: 5 Ci/mmol) at a final concentration of 1 µCi/ml.

Harvesting. Immediately after the pulse, the suspension was centrifuged and the pellet resuspended in 2 ml of hypotonic solution (0.075 M KCl) to facilitate red cell hemolysis. After 10 min the cells were treated with 5 ml of cold ethanol-acetic acid mixture (1 : 1) for 30 min and washed twice in the same mixture so that in the final sediment only white cells, almost exclusively lymphocytes at the 48th and 72nd h, were present. The three replicates for each experimental condition were then pooled to give 1.5 ml of cell suspension in saline, which were used for DNA quantitative determination (0.5 ml), liquid scintillation spectrometry (0.5 ml), and autoradiographic studies (0.5 ml).

DNA Quantitative Assay was made with spectrophotometric method (Burton, 1956; Tedesco and Mellman, 1966). An aliquot of 0.5 ml of the final cell suspension was added with 0.5 ml of 1 N $HClO_4$ and the DNA extracted by incubation at 70°C for 15 min. After cooling, 2 ml of diphenylamine were added to the hydrolysate, and the mixture incubated at 30°C for 20 h. OD was then read at 600 nm, and the amount of DNA calculated using the extinction coefficient obtained from the standard curve done with known concentrations of *E. coli* DNA (Worthington, USA). Reliability and reproducibility of the method were checked by estimating the amount of DNA of white cells and of separated lymphocytes from whole blood or plasma prior to and after the application of the same procedures adopted in our experiments as described above. The average estimate from 36 samples amounted to 11.573 ± 1.030 µg per 1×10^6 cells,

which agrees well with Rudkin's et al. (1964) and Tedesco and Mellman's (1966) estimates, and with the commonly accepted value of 12 picograms per human cell.

Scintillation Spectrometry. An aliquot of 0.5 ml of the final cell suspension was filtered, dried, and dissolved in 2 ml of soluene 100 (Packard, USA) in a scintillation vial. After 12 h, 7 ml of scintillation cocktail—toluene (Merck) 11, PPO (Packard, USA) 4 g, POPOP (Packard, USA) 0.05 g—was added and the activity measured in a Packard Tri-Carb liquid scintillation spectrometer.

Autoradiography. The cells of a third aliquot (0.5 ml) of the final cell suspension were spread upon accurately cleaned glass slides and air dried. Slides were then properly coated with NTB 2 Kodak liquid emulsion (Kodak, USA), maintained in a light- and wet-proof box at 4°C for 5 days, developed in Kodak D 19, stained with Giemsa, and scored at the microscope.

Results

1. Differential Responsiveness of 21-Trisomic and Normal-Diploid Lymphocytes. The dose dependence of the induction of DNA synthesis in PHA-stimulated normal lymphocytes is well known (Hardy and Ling, 1973). Therefore the dose effect has been compared in experimental and control cells. The lymphocyte response has been measured by the amount of ^{3}H-thymidine incorporated into DNA during a pulse labeling of 60 min at the 71st h of incubation, which corresponds to the higher peak of thymidine uptake in short-term lymphocyte cultures (Michalowski, 1963; Hardy and Ling, 1973; Serra and Arpaia, 1975), and it has been expressed as the mean cpm/μg DNA. Since it has been demonstrated (Ziegler et al., 1974) that the ^{3}H-thymidine uptake into DNA of stimulated lymphocyte population follows a log-normal distribution, geometric statistics were adopted for the calculation of the average ^{3}H-thymidine incorporated at each dose. The estimated geometric means are given by $\bar{X}_i = \text{antilog}\left(\frac{1}{n_i}\log\sum_{i,j} X_{ij}\right)$, where X_{ij} represents the observed value of cpm for the sample j at the i est dose, irrespective of age, and n the number of samples studied at the i est dose. They were then plotted in log-log graph paper (Fig. 1). Inspection of the two dose-response curves suggests that, in both types of cell, the response to PHA increases according to a geometric law very rapidly up to the dose of 100 μg/ml and less rapidly from 100 to 600 μg/ml with an optimum at 400—600 μg/ml. But, as the best-fit lines (dotted lines in Fig. 1) evidently show, the responsiveness of 21-trisomic cells is constantly lower than that of normal-diploid cells.

2. Age Effect. The responsiveness of the 21-trisomic lymphocytes seems to decrease with the age of the donor. The regression lines fitting our data (Fig. 2) indicate that, at the optimal PHA dose (400 μg/ml in our experimental conditions), there is an apparently exponential near significant decrease in the ^{3}H-thymidine uptake ($r = 0.34$; $df = 28$; $P = 0.07$). This effect, however, already mentioned by Burgio et al. (1975), requires confirmation by further investigations.

3. Differential Proportions of Responding Cells. The fraction of responding lymphocytes has been estimated from the proportion of labeled cells at the 72nd h

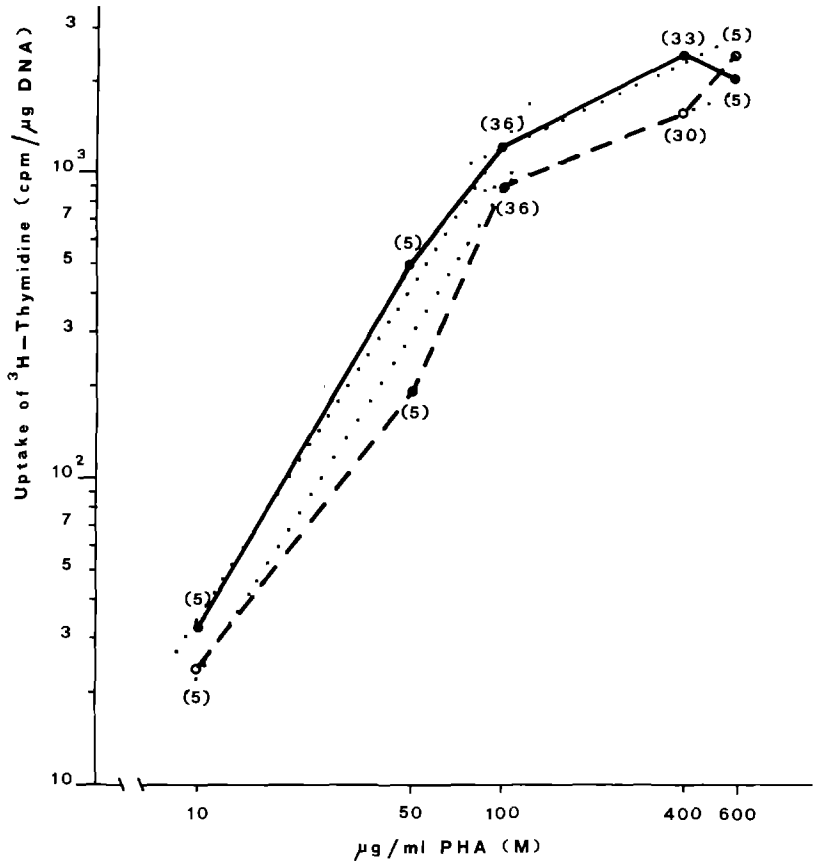

Fig. 1. Experimental dose-response curves and best-fit lines for PHA(M)-stimulated 21-trisomic (-o---o-) and normal-diploid (-●—●-) lymphocytes

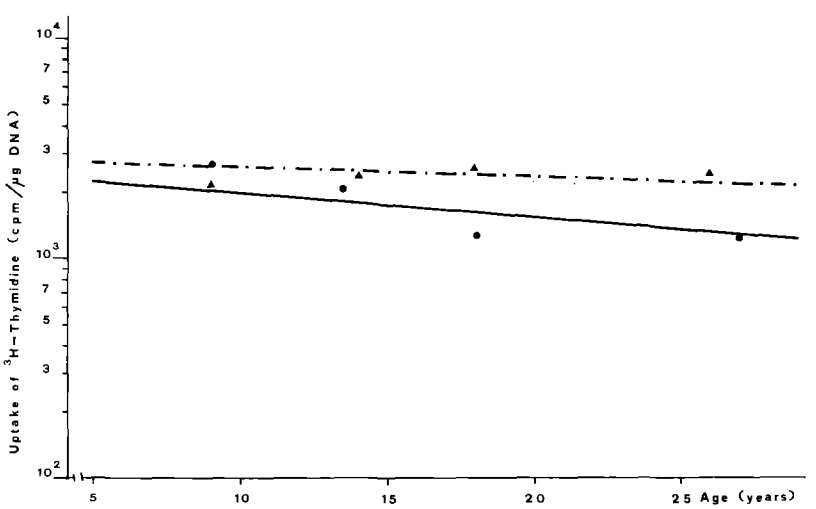

Fig. 2. Regression lines of responsiveness on age for PHA(M)-stimulated 21-trisomic (——, ●) and normal-diploid (—·—·—, ▲) lymphocytes

Table 1. Maximum likelihood estimates of the proportion of labeled cells (P_c) and their span (w_c) according to lymphocyte karyotype, PHA dose, and age

Dose of PHA µg/ml	Age	21-Trisomic lymphocytes			Normal-diploid lymphocytes			χ^2
		No. of subjects	P_c	$\pm w_c$	No. of subjects	P_c	$\pm w_c$	
100	<10	4	0.2610	0.0098	—	—	—	
	11–15	9	0.1976	0.0059	10	0.2686	0.0063	66.11
	16–20	8	0.1850	0.0061	6	0.3507	0.0087	246.15
	>20	8	0.1588	0.0058	4	0.2474	0.0052	344.13
	All ages	29	0.1921	0.0033	30	0.2751	0.0036	282.58
400	<10	6	0.3563	0.0087	3	0.3720	0.0124	0.99
	11–15	9	0.3244	0.0070	13	0.3928	0.0061	53.27
	16–20	5	0.3060	0.0092	5	0.3672	0.0096	20.69
	>20	12	0.3118	0.0060	12	0.4223	0.0064	157.18
	All ages	32	0.3228	0.0037	33	0.3978	0.0038	197.49

Table 2. Maximum likelihood estimates of the proportion of labeled cells (P_c) and their span (w_c) in PHA-stimulated cultures of lymphocytes of 21-trisomic and normal-diploid subjects after different times of incubation at two PHA dose levels

Dose of PHA µg/ml		21-Trisomic lymphocytes			Normal-diploid lymphocytes		
		24 h	48 h	72 h	24 h	48 h	72 h
100	n[a]	13	28	29	18	32	30
	P_c	0.0057	0.1224	0.1921	0.0089	0.1506	0.2751
	$\pm w_c$	0.0009	0.0028	0.0033	0.0010	0.0028	0.0036
400	n	21	27	32	21	29	33
	P_c	0.0064	0.2087	0.3228	0.0085	0.2630	0.3978
	$\pm w_c$	0.0008	0.0035	0.0037	0.0009	0.0037	0.0038

[a] Number of subjects. 500 cells were scored for each subject

of incubation after 1 h pulse with ^{3}H-thymidine. Maximum likelihood estimates have been calculated according to Edwards (1972).

In Table 1 the best-supported values and their span are reported for sub-optimal and optimal PHA doses and for different ages. It is evident, from one side, that the proportion of cells in S phase is dose-dependent, but, from the other side, that at both doses and at each age, unless under ten, the proportion of labeled cells is significantly lower in the 21-trisomic population than in the normal-diploid one. Therefore, after ten years of age, the fraction of responding cells is significantly smaller in the former.

4. Response Delay. The proportions of cells in S phase were also studied at three different times of incubation and are reported in Table 2. Inspection of their

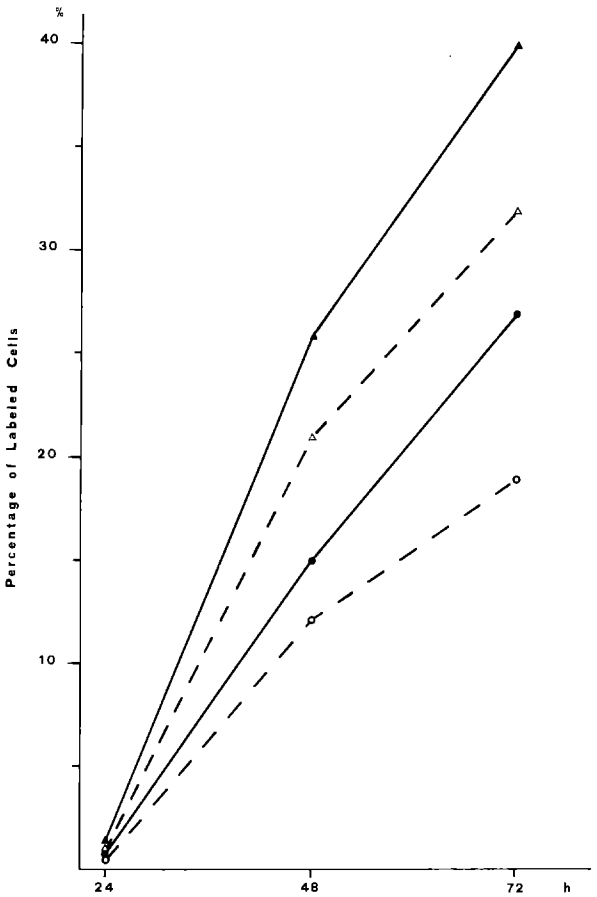

Fig. 3. Increment of the percentage of labeled cells in PHA(M)-stimulated lymphocyte cultures: (-o---o-) 21-trisomic and (-●—●-) normal-diploid lymphocytes at the suboptimal dose of 100 μg/ml; (-△---△-) 21-trisomic lymphocytes and (-▲—▲-) normal-diploid lymphocytes at the optimal dose of 400 μg/ml

increment with time (Fig. 3) in the two cell populations indicates that the portion of 21-trisomic lymphocytes synthesizing DNA increases at a significantly lower rate. While for instance, by the 48th h of incubation at PHA dose of 400 μg/ml, 26% of euploid lymphocytes had entered the S phase, the 21-trisomic ones attained this level only 6 h later.

5. Mean Rate of DNA Synthesis. Let $P(c)$ represent the percentage of cells synthesizing DNA between the 71st and 72nd h of incubation in a given sample, and Q the total amount of DNA, i.e., DNA from labeled and unlabeled cells, spectrophotometrically determined in the same sample. Then, $Q^* = QP(c)$ is the amount of DNA effectively incorporating ^{3}H-thymidine and $I = \text{cpm}/Q^*$ is the measure of the ^{3}H-thymidine incorporated in 1 h pulse per μg of synthesizing DNA. The corresponding estimated means ($\hat{I}$) are given in Table 3. The average amount of

Table 3. Estimates of the average uptake ($\hat{I}$) of ^{3}H-thymidine (cpm/μg DNA) by 21-trisomic and normal-diploid lymphocytes during S phase at two PHA dose levels

Dose of PHA μg/ml	21-Trisomic lymphocytes			Normal-diploid lymphocytes			t	P
	Sample size n	cpm/μg DNA $\hat{I}$	Fiducial limits	Sample size n	cpm/μg DNA $\hat{I}$	Fiducial limits		
100	26	3712	2802–4919	24	3702	2910–4710	0.014	0.90
400	30	4865	4110–5759	32	5764	4831–6884	1.380	0.20–0.10
		$t = 1.665$	$P \doteq 0.10$		$t = 2.867$	$P \doteq 0.01$		

^{3}H-thymidine incorporated by the 21-trisomic synthesizing lymphocytes is not significantly different from that of normal-diploid synthesizing cells. Therefore, assuming that the rate of incorporation of the labeled base reflects the rate of synthesis of DNA, this does not appear to differ in the two types of cell. However, it seems dependent on the dose of PHA.

Discussion

The 21-trisomic lymphocytes are low-responders to PHA. This is shown by the overall decrease of ^{3}H-thymidine incorporation into DNA, compared to the levels attained by normal-diploid lymphocytes. The greater the age of the subject affected with Down's syndrome, the lower seems the lymphocyte responsiveness. Our conclusion agrees with that of Agarwal et al. (1970), who found a significantly lower stimulation of DNA polymerase activity in 3-day cultures of adult (15—22 years old) mongol lymphocytes, running parallel to thymidine uptake measurements, and with that of Hsia et al. (1971) whose studies on the polyribosome profiles of PHA-stimulated mongol lymphocytes clearly indicated an impairment in RNA and DNA response. The disagreement with Fowler and Hollingsworth's data (1973) may be ascribed to the presumably younger age of the subjects in their sample.

At the present stage, the problem of the defect of responsiveness of 21-trisomic lymphocytes is irresolvable, for the factors and mechanisms underlying the response of lymphocyte populations to mitogenic lectins are still obscure. However, several investigations (Michalowski, 1963; Jassinska et al., 1970; Cohen, 1970; Lindhal-Kiessling and Mattson, 1971; Lindhal-Kiessling, 1972; Hardy and Ling, 1973; Phillips and Roit, 1973; Dudin et al., 1974; Greaves et al., 1974; Weber et al., 1974; Skoog et al., 1974; Betel and Van den Berg, 1975) have shown that the kinetics of the proliferative response of mitogen-stimulated lymphocytes reflects a number of variables. Among them, four seem most important. (1) The size of the initial cell population fraction reacting to the mitogen, which can be correlated to the distribution of lymphocyte subpopulation or to binding characteristics of membrane receptors, and enhanced by the recruiting effect of stimulated cells. (2) The duration of induction time of DNA synthesis, i.e., the time of transit from

G_0 into G_1 and from G_1 into the S phase. This duration, on its own side, could vary in consequence of diverse growth properties inherent to different lymphocyte subpopulations. (3) The timing of the cell cycle, which would be reflected in the number of cell divisions. (4) A specific genetic control, which has already been demonstrated, as for the response to PHA and Con A in the mouse (Donner et al., 1973; Heiniger et al., 1975) and chicken (Miggiano et al., 1976). Our data indicate that some of these factors are involved in the overall nuclear response of 21-trisomic lymphocyte population to PHA. The lower level of ^{3}H-thymidine incorporation evidently results from the smaller number of cells that are triggered to proliferate. This is demonstrated by the diminished proportion of DNA-synthesizing cells, associated with an apparently normal average rate of DNA synthesis for those that are triggered. Speculatively this could be correlated to a different distribution of T- and B-cells and/or functionally specific T-cell subpopulations (Cantor and Boyse, 1975; Paul and Benacerraf, 1977) that constitute the PHA-sensitive selective class, though not exclusive (Phillips and Roit, 1973; Greaves et al., 1974; Clot et al., 1975; Potter and Moore, 1975; Chess et al., 1976). Supporting the first hypothesis is the evidence (Burgio et al., 1974, 1975; Levin, 1975) that, in subjects affected with Down's syndrome, the proportion of T-cells is approx. 60% of normal subjects. Supporting the second hypothesis is the apparent absence of correlation between the decreasing responsiveness with age and the number of rosette-forming cells (Burgio et al., 1975). Of course, the variation of responsiveness with age could well depend on the changing of lymphocyte population composition with time. The smaller labeling index of cultured 21-trisomic cells may, however, also be due to a greater delay in the induction of cell to initiate its cycle. A basis for this assumption is provided by the slower increment of labeled 21-trisomic cells with time, shown in our experiments, suggesting a longer mean transit time, i.e., a retardation in the multistep sequence of processes that make the cell move from G_0 to G_1 and S phase. At what step or steps disturbance occurs capable of provoking this retardation cannot presently be stated. Recent advances in the analysis of factors and mechanisms implicated in the process of proliferation of lectin-stimulated lymphocytes have definitively proved that the first essential step in the chain of events is the binding of mitogen molecules to proper membrane receptors (Younkin, 1972; Cunningham et al., 1976; Dutton, 1976; Novogrodsky, 1976; Wang et al., 1976). It has also shed some light on surface and intracellular events, e.g., Ca^{++}, AMP, GMP, and GTP changes and adenylate cyclase activation, strictly correlated with the interaction of the ligand with its receptor and undoubtedly important for the transfer of signals to the nucleus (Cohn, 1976; Crumpton, 1976; Wang et al., 1976; Byus et al., 1977; Hesketh et al., 1977). This new accumulating knowledge obviously suggests possible lines of experimental attack to investigate the observed 21-trisomic lymphocyte deficiency.

Testing membrane receptor properties with probes other than PHA and an accurate cell cycle analysis seemed two lines worth following for their informative potential, although they would still be of a simple descriptive type. The results will be communicated elsewhere.

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