

# DNA Polymerase Activity as an Index of Lymphocyte Stimulation: Studies in Down's Syndrome

S. S. AGARWAL, BARUCH S. BLUMBERG, BETTY JANE S. GERSTLEY,  
W. THOMAS LONDON, ALTON I. SUTNICK, and LAWRENCE A. LOEB

*From The Institute for Cancer Research, Fox Chase,  
Philadelphia, Pennsylvania 19111*

**ABSTRACT** The ability of peripheral blood lymphocytes to respond to phytohemagglutinin (PHA) in vitro was studied in patients with Down's syndrome. The response was measured by the increase in DNA polymerase activity and the rate of incorporation of tritiated thymidine by the cultured lymphocytes. These activities were significantly lower in PHA-stimulated lymphocytes from patients with Down's syndrome compared with age- and sex-matched, mentally retarded patients without Down's syndrome from the same institution and the normal healthy volunteers. The impairment in response to PHA does not seem to be related to the presence of Australia antigen in patients with Down's syndrome or to institutionalization itself. In contrast to DNA polymerase activity and thymidine-<sup>3</sup>H uptake, there was no significant difference in the percentage of blast transformation in the three groups studied. The poor response of the lymphocytes from patients with Down's syndrome to a mitogenic stimulus could reflect an impairment of cellular immune functions in these patients which may be one of the factors contributing to the vulnerability of these patients to repeated or persistent infections.

## INTRODUCTION

Impairment of immunologic defenses in patients with Down's syndrome can be inferred from a number of clinical and epidemiological studies. Several authors have reported increased susceptibility of these patients to infections, particularly those of the respiratory tract (1-3). Institutionalized patients with Down's syndrome also have a high frequency of Australia antigen associated with chronic anicteric hepatitis (4). Furthermore,

these patients have been shown to have a high prevalence of thyroid autoantibodies (5, 6) and a much higher risk of developing leukemia than those in the general population (7, 8). It is possible that defects in the immune shield may not only make these patients vulnerable to infectious agents but to allergens and carcinogens as well. Although there is evidence of immune deficiencies in patients with solid tissue tumors and leukemia, there is no direct evidence that abnormal immune responsiveness precedes the development of these malignancies (9, 10). The high susceptibility of patients with Down's syndrome to leukemia is of particular significance, since it provides an opportunity to study immunological functions before the onset of clinical leukemia in a well defined group.

The in vitro lymphocyte stimulation test has been used by several workers to evaluate cellular immunity (11, 12). The ability of lymphocytes to respond to a mitogen is commonly measured by observing the morphological transformation of these cells into large blast-like cells (blastogenesis) and by measuring the amount of radioactive thymidine incorporated into cellular DNA by these cells. We recently reported that addition of phytohemagglutinin (PHA) to cultures of human peripheral lymphocytes results in a 30- to 100-fold increase in DNA polymerase activity and that it could be a more reliable parameter of mitogenic response (13). In the present study we have examined the utility of this parameter along with the measurement of radioactive thymidine incorporation and cytological observations to monitor the lymphocyte stimulation. Results from the patients with Down's syndrome are compared with those from other mentally retarded patients without Down's syndrome and from normal controls. The findings are discussed in relation to the presence of Australia antigen in patients with Down's syndrome, other immunological functions and genetic constitution of these patients.

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## METHODS

**Selection of patients.** The studies were carried out on lymphocytes from 12 patients with Down's syndrome (2 females, 10 males) from Pennhurst State School and Hospital, Spring City, Pa. Six of these patients had Australia antigen in their peripheral blood, and six did not. The diagnosis of Down's syndrome was confirmed by karyotype analysis; all the patients had trisomy of the G group. Simultaneously, studies were done on lymphocytes from six mentally retarded children without Down's syndrome from the same institution (one female, five males) and six normal healthy volunteers from our staff. All six mentally retarded patients without Down's syndrome were karyotypically normal. Written permission was obtained from the parents of all mentally retarded patients for these studies. All persons were carefully age and sex matched. The age range of patients with Down's syndrome was between 14 and 22 yr, with a mean of 17.6 yr. The ages of other mentally retarded patients without Down's syndrome ranged from 16 to 18 yr, with a mean of 17.1 yr; the normal controls were between 18 and 22 yr of age.

In addition to these matched and planned groups, four patients with Down's syndrome showing varying degrees of mosaicism in peripheral blood and one patient with Down's syndrome having D/G translocation [46, XY, D-, G=t(DqGq)+], have also been studied. Both parents of the child with translocation were also studied. The mother of the child is the translocation carrier.

**Clinical and laboratory studies.** All the patients were admitted to our Clinical Research Unit. Clinical evaluation emphasized the history of past infections, immunizations, and evidence of present infection. Laboratory examination included total blood counts and serum protein electrophoresis. Quantitative determination of immunoglobulin levels was carried out by single radial diffusion in agar plates (Hyland Laboratories, Los Angeles, Calif.). Incubation was for 16 hr at 25°C for determination of IgA and IgM. IgG was determined after incubating 4 hr at 37°C. Serum from all persons was tested for the Australia antigen by the micro-Ouchterlony technique. Cutaneous sensitivity was tested to the following antigens: 0.1 ml of mumps antigen (Eli Lilly & Co., Indianapolis, Ind.), 0.1 ml of Schick's antigen (Wyeth Laboratories, Marietta, Pa.), 0.1 ml of intermediate strength P.P.D.<sup>1</sup> (Merck Sharp & Dohme, West Point, Pa.), 0.1 ml of histoplasmin (Parke, Davis & Company, Detroit, Mich.), 0.05 ml of Dermatophytin "O" (fungus extract of *Oidiomyces* [*Candida albicans*] from Hollister-Stier Laboratories, Yeadon, Pa.), 0.05 ml of Trichophytin (Hollister-Stier Laboratories), and 0.1 ml of streptokinase-streptodornase-varidase (Lederle Laboratories, Pearl River, N. Y.). The stated amounts of antigens were injected intradermally, and at 24, 48, and 72 hr the degree of erythema and induration at the site of injection was measured. Only an induration of 5 × 5 mm or greater persistent at 72 hr was scored as a positive reaction.

**Preparation of lymphocyte cultures.** Lymphocytes were obtained from 50 ml of heparinized (50 U/ml) venous blood by the method of Bach and Hirschorn (14). The final preparation of lymphocytes was suspended at a concentration of  $7.5 \times 10^6$  cells/ml in Eagle's MEM (Spinner modification) (GIBCO, Grand Island, N. Y.), with 20% fetal calf serum, 1% l-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). 2-ml portions of the cell suspension were incubated with 0.05 ml of PHA-M (General Biochemical

Div., North American Mogul Products Co., Chagrin Falls, Ohio, lot No. 685,291) in 15 ml Corex test tubes at 37°C for 3 days. All reagents used in this study were from the same lot.

**Incorporation of thymidine.** 2 hr before harvesting of the cultured cells, 5.0 µc of methyl thymidine-<sup>3</sup>H (Schwarz Bio-Research, Orangeburg, N. Y., 11 Ci/mmole) was added to each culture. Incorporation of labeled thymidine was terminated by adding 10 µmoles of unlabeled thymidine to each culture and placing the cultures in an ice-water bath. The cells were then washed by centrifugation for 10 min at 2500 g with 0.15 M potassium chloride. To the final pellet was added 1 ml of a solution made up of 20% (w/v) glycerol (Matheson, Coleman & Bell, East Rutherford, N. J., Spectroquality grade), 0.02 M potassium phosphate buffer (pH 7.4), 0.001 M potassium EDTA and 0.004 M reduced glutathione. Thereafter the cells were disrupted by alternate rapid freezing and thawing. The amount of radioactivity incorporated into acid-insoluble material was determined along with the assay of DNA polymerase activity.

**Assay of DNA polymerase activity.** The assay for DNA polymerase activity measures the incorporation of an appropriately labeled deoxynucleotide into an acid-insoluble product. The reaction mixture in a total volume of 0.3 ml, is the following: 25 µmoles Tris (hydroxymethyl) amino-methane-maleate buffer, pH 8.0; 3 µmoles magnesium chloride; 1 µmole potassium chloride; 0.3 µmoles β-mercaptoethanol; 25 µmoles each of dATP, dCTP, dGTP, (California Foundation for Biochemical Research, Los Angeles), and 10 µmoles of α-<sup>32</sup>P-dTTP (about  $5 \times 10^4$  dpm/µmole) (International Chemical & Nuclear Corp., City of Industry, Calif.); 266 µmoles of "activated" calf thymus DNA and 0.1 ml of the lymphocyte preparation (13).

Incubation was for 1 hr at 37°C and the reaction was stopped by adding 0.5 ml of cold 1 M perchloric acid containing 0.01 M sodium pyrophosphate. With each set of reaction mixtures a known amount of purified sea urchin nuclear DNA polymerase was assayed simultaneously. The acid-insoluble material was collected on glass-fiber filters (15). Radioactivity was determined by standard dual labeling techniques using liquid scintillation spectroscopy.

**Assay of morphological transformation.** Cytological analysis was carried out in separate simultaneous lymphocyte cultures. Cell viability was determined by the Trypan Blue dye exclusion technique (16). The extent of blast transformation and mitosis was determined by adding 0.1 ml of Velban<sup>2</sup> (0.5 µg/ml) to designated 2 ml cultures. 2 hr later the cells were fixed in acetic-methanol (1:3) and air-dried preparations (17) were stained with Giemsa. From each culture, a total of 1000 cells were counted and grouped into small lymphocytes, lymphoblasts, and cells in mitosis.

## RESULTS

The study was designed to evaluate lymphocyte function in patients with Down's syndrome and to ascertain whether there is any relationship with the presence of Australia antigen, Au(1), in these patients. Our preliminary observations showed that, even in normal healthy volunteers, there might be considerable day-to-day variation in the response of lymphocytes to phytohemagglutinin. For this reason the tests were carried out on age- and sex-matched pairs and the results were

<sup>1</sup> P.P.D. = tuberculin-purified protein derivative.

<sup>2</sup> Eli Lilly & Co., Indianapolis, Ind.

statistically evaluated with the use of Wilcoxon's matched-pairs signed-ranks test (18). The total study included six age- and sex-matched quartets. Each quartet consisted of a patient with Down's syndrome with Australia antigen, a patient with Down's syndrome without Australia antigen, a mentally retarded patient without Down's syndrome from the same institution, and a normal healthy volunteer. The lymphocytes from each patient were cultured in triplicate in the presence of PHA. Determinations of DNA polymerase activity and thymidine uptake in the triplicate assays were in fair agreement ( $\pm 10\%$ ). The experiment on each quartet was repeated at least once on another day in order to test whether the differences were in the same direction. The results of each quartet were sequentially analyzed (19) to decide when to stop the study.

**Optimum concentration of PHA.** The lymphocytes from a quartet were cultured with different concentrations of PHA. The response of a normal healthy volunteer and a patient with Down's syndrome, as a function of the amount of PHA added to the culture, is shown in Table I. The amount of PHA routinely used is designated as  $1 \times$  which corresponds to  $50 \mu\text{g}/1.5 \times 10^6$  lymphocytes in a 2 ml culture. The response to  $1 \times$  and  $2 \times$  concentrations is essentially the same both in the normal individual and in the patient with Down's syndrome, except that the DNA polymerase activity is higher with  $1 \times$  concentration in cultures from the normal person. In contrast, the lower concentrations ( $1:10$  and  $1:25$ ) and the higher concentration ( $5 \times$ ) evoked significantly lower responses than those obtained with  $1 \times$  concentration. There was no detectable response to  $1:100$  and  $1:50$  concentrations of PHA in all four members of the quartet. We did not find any differences in the

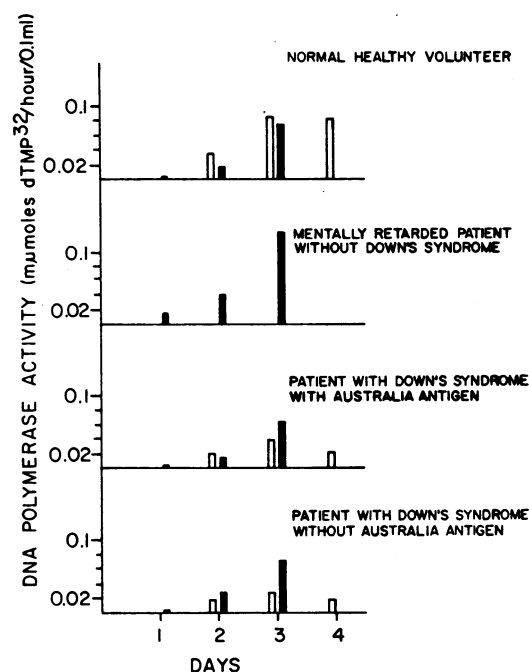


FIGURE 1 Cultures of human peripheral lymphocytes were obtained from the patients indicated. The cultures were terminated on the days indicated and DNA polymerase activity was assayed as given in Methods.

optimal concentrations of PHA in the different groups, with DNA polymerase activity and thymidine incorporation as the parameters of the mitogenic response to PHA. Therefore, in all our studies we have used the designated  $1 \times$  concentration of the PHA-M.

**Duration of incubation of cultures.** Differences in response at any given point may be due to differences in the kinetics of the *in vitro* response in different cultures. For this reason cultures from a quartet were incubated at  $37^\circ\text{C}$  for different lengths of time. In one experiment the cultures were harvested on days 1, 2, and 3 and in another experiment on days 2, 3, and 4. The results are shown in Fig. 1. It is evident that the largest increase in DNA polymerase activity was obtained after 3 days in culture in all the members of the quartet. Similar results were obtained for the ability of the cells to incorporate labeled thymidine into DNA. All studies were therefore carried out on cultures incubated for 3 days at  $37^\circ\text{C}$ .

**Impairment of lymphocyte stimulation in Down's syndrome.** A direct comparison of the ability of lymphocytes to respond to PHA between patients with Down's syndrome and age- and sex-matched normal volunteers shows a significantly lower stimulation of DNA polymerase activity in Down's syndrome. In all but 4 of the 30 comparisons on 12 pairs, the response was higher in the normal controls (Table II, compare columns 3

TABLE I  
Effect of Different Amounts of PHA on  
Lymphocyte Transformation

PHA concn	DNA polymerase activity		Thymidine- $^3\text{H}$ uptake	
	Normal	Down's syndrome	Normal	Down's syndrome
	nmol dTMP/hr per 0.1 ml		cpm/0.1 ml	
1:25	0.002	0.009	117	167
1:10	0.018	0.016	1183	318
$1 \times$	0.091	0.052	8968	4313
$2 \times$	0.066	0.060	9089	4284
$5 \times$	0.028	0.021	4726	1756

DNA polymerase activity and incorporation of thymidine were determined as given in Methods. The standard concentration of PHA ( $1 \times$ ) corresponds to  $0.05 \text{ ml/culture}$ . Dilutions were made in distilled water.  $2 \times$  and  $5 \times$  refer to  $0.1$  and  $0.25 \text{ ml}$  of standard PHA solution.

and 4 with column 1). A sequential analysis (19) of these results is shown in Fig. 2a; the response of lymphocytes to PHA stimulation is greater in normal controls. Analysis by Wilcoxon's matched-pairs signed-ranks test gives a  $P$  value of  $< 0.001$ . The ability of the same cultures to incorporate thymidine into DNA was also determined and might be considered either a related or possibly an independent parameter of lymphocyte transformation. The results are nearly identical; there is a much lower incorporation of thymidine in cultures of lymphocytes obtained from patients with Down's syndrome (Table III, compare columns 3 and 4 with column 1). In 30 comparisons the values in the normals were higher than in patients with Down's syndrome in all except six cases.  $P$  value by Wilcoxon's matched-pairs signed-ranks test is 0.02. In contrast to these biochemical determinants, simultaneous cytological analysis of the cultures revealed no significant difference ( $P > 0.05$ ) in the per cent of lymphocytes transformed

TABLE II  
DNA Polymerase Activity

Exp. No.	Normal	NDMR*	DS(Au <sub>1</sub> )‡	DS(Au <sub>0</sub> )§
<i>Residues of dTMP incorporated <math>\mu</math>moles/hr per 0.1 ml</i>				
I	1	0.101	0.241	0.005
	2	0.167	0.151	0.009
	3	0.091	0.185	0.006
II	1	0.117	0.172	0.152
	2	0.105	0.138	0.062
III	1	0.097	0.054	0.004
	2	0.091	0.068	0.043
IV	1	0.093	0.029	0.010
	2	0.051	0.005	0.007
V	1	0.086	—	0.036
	2	0.031	—	0.009
	3	0.019	0.041	0.142
VI	1	0.145	0.164	0.041
	2	0.217	0.071	0.121
	3	0.094	0.261	0.056

Lymphocytes from four age- and sex-matched patients were cultured simultaneously. Six such quartets (I–VI) were studied. Only the highest of the three values from a set of triplicate cultures from each experiment is given. Experiments on quartets II, III, and IV were repeated once and on quartets I, V, and VI were repeated twice on separate days.

\* NDMR: mentally retarded patient without Down's syndrome.

‡ DS(Au<sub>1</sub>): patient with Down's syndrome having Australia antigen.

§ DS(Au<sub>0</sub>): patient with Down's syndrome without Australia antigen in his serum.

TABLE III  
Thymidine-<sup>3</sup>H Uptake

Exp. No.	Normal	NDMR*	DS(Au <sub>1</sub> )‡	DS(Au <sub>0</sub> )§
<i>cpm/0.1 ml</i>				
I	1	7,696	11,265	360
	2	13,461	13,095	1,347
	3	11,542	7,918	293
II	1	9,543	10,339	9,772
	2	7,911	11,756	10,390
III	1	11,989	10,804	189
	2	9,543	10,704	3,421
IV	1	12,100	4,603	856
	2	5,156	1,379	536
V	1	5,500	—	1,357
	2	1,538	—	717
	3	2,748	4,395	7,007
VI	1	11,881	6,610	4,507
	2	6,389	8,608	1,018
	3	4,215	9,733	4,260

\* NDMR: mentally retarded patient without Down's syndrome.

‡ DS(Au<sub>1</sub>): patient with Down's syndrome having Australia antigen.

§ DS(Au<sub>0</sub>): patient with Down's syndrome without Australia antigen in his serum.

from patients with Down's syndrome and from the normal controls (see Table IV).

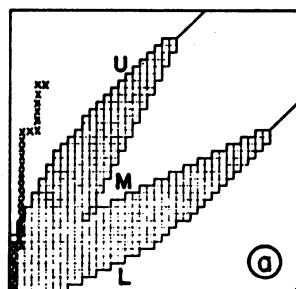
*Comparison with other mentally retarded children.* In order to evaluate the possible contribution of host and environmental factors on the extent of lymphocyte stimulation in patients with Down's syndrome, age- and sex-matched mentally retarded patients without Down's syndrome from the same institution were included in each experimental quartet. Results of the DNA polymerase activity are shown in Table II (compare columns 3 and 4 with column 2) and the extent of thymidine incorporation is shown in Table III (compare columns 3 and 4 with column 2). There is significantly lower DNA polymerase activity ( $P < 0.001$ ) and thymidine incorporation ( $P < 0.001$ ) in cultures from patients with Down's syndrome than in cultures from mentally retarded patients without Down's syndrome. Sequential analysis of the results is shown in Fig. 2c. Here again the response to PHA in mentally retarded patients without Down's syndrome is greater than in Down's syndrome. The results are similar to the ones obtained by comparison of the patients with Down's syndrome to normal healthy volunteers. Furthermore, there is no significant difference between mentally retarded patients without Down's syndrome and the normal controls ( $P > 0.05$ ).

This shows that the institutionalization per se is not responsible for the differences in lymphocyte stimulation as seen in patients with Down's syndrome (Fig. 2 b).

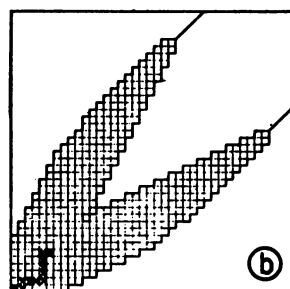
*Australia antigen and patients with Down's syndrome.* The association of Australia antigen with hepatitis (20) and its persistence in some patients with Down's syndrome can be used to study the possible relationship between lymphocyte stimulation and the presence of chronic hepatitis. When the level of DNA polymerase activity and the uptake of thymidine are considered in

relation to the presence or absence of Australia antigen in patients with Down's syndrome (compare columns 3 and 4 in Tables II and III), we do not find any significant differences. *P* values for both, by Wilcoxon's matched-pairs signed-ranks test, are  $> 0.05$ . The data are insufficient for a definite answer by sequential analysis but the trend suggests that there is no difference between the two groups (Fig. 2 d).

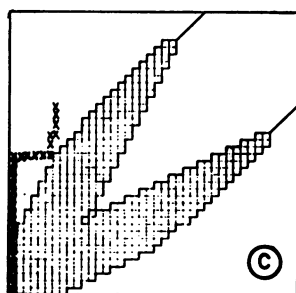
*Relationship of lymphocyte stimulation to immunoglobulin levels.* Total proteins, gamma globulins and



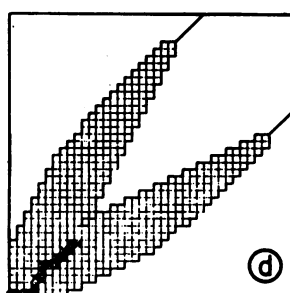
Normals (A) compared with patients with Down's syndrome (B)



Normals (A) compared with mentally retarded patients without Down's syndrome (B)



Mentally retarded patients without Down's syndrome (A) compared with patients with Down's syndrome (B)



Patients with Down's syndrome having Australia antigen (A) compared with patients with Down's syndrome without Australia antigen (B)

FIGURE 2. Results of sequential analysis based on pair differences (19). Significance probability  $\alpha = 0.05$ . Lymphocytes from both the members of the pair (designated A and B as above) were cultured in presence of PHA simultaneously and assayed for DNA polymerase activity. If the activity in A was higher than B, a cross was made immediately above the black square in the chart. If the activity in B was higher than A, a cross was made in the square immediately to the right of the black square. If there is no difference, no entry is made. A second test was made in exactly the same way and the result was entered in the square above or to the right of that marked in the first test and so on for successive tests. Results of repeat experiments on the same group are also plotted separately each time. As soon as the barrier is overstepped one of the following decisions is indicated: (1) upper barrier (U) overstepped, response in A is higher than in B; (2) lower barrier (L) overstepped, response in B is higher than in A; (3) middle barrier (M) overstepped, no difference demonstrated.

TABLE IV  
*Lymphoblast Transformation*

Exp.	Per cent lymphoblast transformation			
	Normal	NDMR*	DS(Au) <sup>†</sup>	DS(Au) <sup>‡</sup>
			%	
I	66	52	48	54
II	57	45	67	43
III	62	79	74	59
IV	58	—	70	68
V	48	52	85	63
VI	87	85	91	66

\* NDMR: mentally retarded patient without Down's syndrome.

<sup>†</sup> DS(Au)<sub>1</sub>: patient with Down's syndrome having Australia antigen.

<sup>‡</sup> DS(Au)<sub>0</sub>: patient with Down's syndrome without Australia antigen in his serum.

amounts of IgA, IgG, and IgM were determined in serum from all patients with Down's syndrome and mentally retarded patients without Down's syndrome. The results are shown in Table V. There are no significant differences in total serum proteins, gamma globulins, IgA, and IgG levels between the two groups of patients ( $P > 0.05$ ). However, the IgM values are significantly lower in patients with Down's syndrome compared with mentally retarded patients without Down's syndrome ( $P < 0.01$ ). In order to find any correlation between the lymphocyte stimulation and specific immunoglobulin levels, we have calculated correlation coefficients ( $r$  values). The results are given in Table VI. A

TABLE VI  
*Correlation of Parameters of Lymphocyte Stimulation with Immunoglobulin Levels*

	Correlation coefficients ( $r$ value)		
	IgA	IgG	IgM
DNA polymerase activity	0.36	-0.03	0.30
Thymidine uptake	0.32	-0.01	0.47

The  $r$  values for IgM and thymidine uptake are significant at the 0.05 level, but not for the other comparisons.

positive correlation coefficient is found for IgA and IgM with the parameters of lymphocyte stimulation. However, the values are not statistically significant ( $P > 0.05$ ) except for thymidine uptake and IgM levels ( $P < 0.05$ ).

*Skin tests.* Delayed cutaneous hypersensitivity was tested for the antigens listed in the methods section. 6 of the 12 patients with Down's syndrome did not react to any of the antigens while only one of the five mentally retarded patients without Down's syndrome did not react. There was no distinct pattern of reactivity to different antigens in the two groups. As most patients reacted to one or two antigens only, our data are not sufficient to determine the correlation between skin reactivity and lymphocyte stimulation.

*Results of studies on other patients.* In one patient with Down's syndrome with D/G translocation the response to PHA stimulation was significantly lower than that in the normal control. These findings are similar to those seen in other trisomic mongols. The mother of

TABLE V  
*Serum Proteins and Immunoglobulin Levels in Patients with Down's Syndrome as Compared with Mentally Retarded Patients without Down's Syndrome*

Exp.	Mentally retarded patients without Down's syndrome					Patients with Down's syndrome				
	Total proteins	$\gamma$ -globulins	IgA	IgG	IgM	Total proteins	$\gamma$ -globulins	IgA	IgG	IgM
	g/100 ml	g/100 ml		mg/100 ml		g/100 ml	g/100 ml		mg/100 ml	
I	7.7	2.17	450	1730	370	7.3	1.77	290	2250	100
II	8.2	1.35	700	2000	200	7.1	1.34	570	2100	165
						7.2	1.51	340	900	90
III	7.7	1.34	240	1500	175	8.4	1.57	300	1500	90
						7.1	0.79	180	850	54
IV	9.2	1.56	150	1550	320	8.6	2.27	220	1900	75
						7.3	1.35	65	1000	200
V	7.5	2.02	210	1650	165	7.8	1.29	390	1200	100
						8.0	1.92	340	950	62
VI	7.3	1.55	125	800	220	8.3	1.65	390	1850	40
						8.3	1.42	205	1250	65
						8.3	1.31	205	900	120

Total gamma-globulins determined by electrophoresis. IgA, IgG, and IgM determined by single radial diffusion technique.

TABLE VII  
Response of Lymphocytes to PHA from a Patient with  
Down's Syndrome with D/G Translocation  
and His Parents

	DNA polymerase activity	Thymidine uptake
	dTMP <sup>32</sup> P $\mu$ moles/hr per 0.1 ml	cpm/0.1ml
Normal	0.073 $\pm$ 0.006	5590 $\pm$ 539
Patient	0.010 $\pm$ 0.015	698 $\pm$ 992
Mother*	0.009 $\pm$ 0.004	719 $\pm$ 288
Father	0.070 $\pm$ 0.011	2891 $\pm$ 785

\* Mother of the patient is a translocation carrier.

this patient is the carrier of the trait and she had a similarly low response whereas the response in the father was normal (Table VII). In contrast, in four patients with Down's syndrome with varying degrees of mosaicism in peripheral blood the degree of stimulation of DNA polymerase activity and thymidine uptake were essentially the same as in normal controls.

## DISCUSSION

This study shows that the lymphocytes of patients with Down's syndrome are markedly unresponsive to PHA stimulation in vitro as compared with normals and other mentally retarded patients without Down's syndrome. Statistically, these differences are highly significant. This unresponsiveness does not appear to be a function of the concentration of PHA or the duration of incubation of the cultures up to 4 days. PHA agglutinates lymphocytes from patients with Down's syndrome as effectively as those from the normals. We have not found any differences in the viability of cells in culture at 66 hr as determined by Trypan Blue dye exclusion technique. Impairment of the lymphocyte response does not appear to be mediated by the patients' serum since the lymphocytes were washed twice before culturing. Also, the comparison with mentally retarded children without Down's syndrome from the same institution indicates that the impairment of lymphocyte stimulation in patients with Down's syndrome is not due to a nonspecific environmental factor prevalent in the institution. For these reasons the impaired responsiveness seems to be an innate characteristic of the lymphocytes of patients with Down's syndrome.

In the present study we did not find a correlation between the morphological changes and the rate of thymidine-<sup>3</sup>H incorporation in PHA-stimulated lymphocytes from patients with Down's syndrome. While the thymidine uptake by the lymphocytes from patients with Down's syndrome was significantly lower than those from normals, there was no difference in the percentage

of lymphoblast transformation. This can be taken to indicate that simultaneous DNA synthesis is not essential for the appearance of morphological changes. Recently, Kay, Levanthal, and Cooper (21) reported that lymphocytes cultured in the presence of actinomycin D which prevents DNA synthesis and mitosis, exhibit typical cytological changes indicative of blastogenesis. Our unpublished observations support their findings. However, the rate of thymidine incorporation may be subject to fluctuations in the concentrations of metabolites within the cell as well as by the activities of the enzymes on the "salvage pathway" by which the external thymidine is incorporated into cellular DNA. In this regard a simultaneous assay of the DNA polymerase activity on the same cultures may not only provide a confirmatory evidence for the validity of thymidine uptake results but may also provide a more reliable parameter to evaluate the true mitogenic response. The addition of PHA to cultures of human lymphocytes results in a marked increase in DNA polymerase activity which is closely related temporally and in magnitude to thymidine incorporation into cellular DNA by the same cultures (13). The activity of the enzyme is assayed in vitro using the cell lysate and known concentrations of substrates. The sensitivity of the assay is only limited by the specific activity of the radioactive precursor. Further the two parameters are independent measurements of the ability of the cells to synthesize DNA. In the present study the DNA polymerase activity and thymidine uptake measurements run parallel in all the lymphocyte cultures from patients with Down's syndrome. Thus the lymphocytes from patients with Down's syndrome are truly hyporesponsive to the mitogenic stimulus of PHA. This disparity between the morphological changes and biochemical events following the PHA stimulation of lymphocytes from patients with Down's syndrome may explain the differences in our results from those reported by Hayakawa, Matsui, Higurashi, and Kabayashi (22) and Sasaki and Obara (23).

The immune status of patients with Down's syndrome has not been systematically studied. Donner (2) found a significantly lower number of tuberculin-positive individuals among patients with Down's syndrome compared with other children from the same locales. However, vaccination with Calmette-Guérin bacillus (BCG) evoked a similar response in patients with Down's syndrome and normal controls. Impairment of antibody formation and functions has not been conclusively demonstrated. The total amount of gamma globulin appears to be increased (24, 25). Sutnick, London, and Blumberg (26) have reported a lower level of immunoglobulin M in outpatients with Down's syndrome. In the present study also, the immunoglobulin M levels are significantly lower in patients with Down's syndrome as

compared to mentally retarded children without Down's syndrome from the same institution and there is a positive correlation between lymphocyte stimulation and IgM levels. The biological significance of this correlation is not known. Conflicting results have been reported as to the ability of patients with Down's syndrome to produce specific antibodies after activity immunization (27, 28). In view of the uncertainties about defects in the humoral antibody in patients with Down's syndrome, the impairment of lymphocyte stimulation could be particularly germane to the clinical observations.

The essential role of circulating lymphocytes in immune responses is well documented (29). PHA-induced in vitro lymphocyte stimulation has been used to assess the immune capabilities of these cells (11, 12, 30, 31). When lymphocytes are cultured in the presence of PHA there is a sequential stimulation of RNA, protein, and DNA synthesis. Simultaneously these cells also undergo morphologic changes resulting in large cells with prominent nucleoli and intensely basophilic cytoplasm. Cells of similar appearance are observed in the regional lymph nodes after *in vivo* immunization (32). Changes similar to those that occur on PHA stimulation are observed on stimulating lymphocytes with antigens to which a person is known to have been sensitized previously (33, 34) and in mixed leukocyte reactions where histocompatibility antigens appear to be the stimulating factors (14). PHA-treated lymphocytes have been shown to synthesize immunoglobulins (35-37), specific antibodies (36, 37), and interferon (38). PHA-stimulated cells are also capable of destroying human fibroblasts in vitro (39). In a number of diseases in which immune deficiency is present an impairment in PHA stimulation of lymphocytes has been reported (for references, see 12). Thus it is reasonable to assume that impaired in vitro lymphocyte stimulation in patients with Down's syndrome reflects an impairment in cellular defenses which could contribute to their enhanced vulnerability to pathogens.

In other studies we have shown a high incidence of Australia antigen, a particle of 200 Å in diameter, associated with chronic anicteric hepatitis in institutionalized patients with Down's syndrome (4). Willems, Melnick, and Rawls (40) have reported impaired response to PHA stimulation in lymphocytes from patients with infectious hepatitis. The presence of a virus in these cells could be responsible for the impairment in the response to a mitogenic stimulus as has been shown by Olson et al. (41) and Willems et al. (40). However, in this study a comparison of the extent of impaired lymphocyte stimulation in groups with Down's syndrome with and without Australia antigen revealed no significant differences. However, the precipitin test used for detection of the Australia antigen may not be

sensitive enough to detect all persons with Australia antigen.

The induction of replication may also be dependent on the genetic constitution of the cells. Kamin, Fudenberg, and Douglas (42) have reported impaired in vitro lymphocyte transformation in the asymptomatic parents of patients with "acquired" agammaglobulinemia. Although the exact role of an extra chromosome in the manifestations of Down's syndrome is not understood at present, it is beyond question that a definite genetic abnormality including a gross chromosomal error exists in these patients. In one translocation mongol (D/G) we have seen impairment in lymphocyte stimulation similar to that observed in mongols with simple G group trisomy. In contrast, the lymphocytes from four patients with Down's syndrome having varying degrees of mosaicism in the peripheral blood responded to PHA stimulation as did the normal controls. However, impaired lymphocyte stimulation to PHA in patients with Down's syndrome may not be a simple attribute of extra chromosomal material. In the mother of the translocation mongol, who is the carrier of the trait, the lymphocytes were equally unresponsive to PHA. It is possible that further studies in this direction may help in understanding the effect of chromosomal aberrations on cell replication.

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