Lymphocyte Transformation and Hepatitis
I. Impairment of Thymidine Incorporation and DNA Polymerase Activity[1]
(35818)

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Proliferation and differentiation of lymphocytes in response to antigenic stimuli are important in the expression of the immunological functions of these cells. *In vitro* transformation of human peripheral blood lymphocytes into actively replicating blast cells provides a model system for studying these phenomena *in vitro*. Certain viruses have been shown to interfere with the initiation of DNA replication during lymphocyte transformation. For example in patients with congenital rubella (1), infectious mononucleosis (2) and acute upper respiratory tract viral infections (3), lymphocytes have been found to be hyporesponsive to mitogenic stimuli *in vitro*. Addition of certain live viruses such as rubella virus and polio virus (1, 4-6) to cultures of phytohemagglutinin (PHA)-stimulated lymphocytes from normal healthy subjects can also inhibit the induction of DNA synthesis. We have started a systematic study of the effect of PHA stimulation on the lymphocytes from patients with diseases associated with Australia antigen [Au(1)], a particle which may be causally related to viral hepatitis (7).

Earlier we reported that lymphocytes from patients with Down's syndrome are markedly hyporesponsive to PHA (8). Many of these patients have Australia antigen in their peripheral blood and an associated chronic anicteric hepatitis (9). In this paper we present quantitative evidence of impairment in DNA synthesis in PHA-stimulated lymphocytes from patients with viral hepatitis which is in accord with the findings of Mella and Lang (10) and Willems *et al.* (5). The following paper concerns the effect of purified Au(1) (11) on the responsiveness of normal lymphocytes to PHA stimulation.

Materials and Methods. Peripheral blood lymphocytes from 9 patients with viral hepatitis and age- and sex-matched normal healthy volunteers were used in this study. The diagnosis of viral hepatitis was made clinically and on the basis of liver function tests. In 3 patients the diagnosis was also confirmed histologically. At some time during the course of the disease, Au(1) was detected by the immunodiffusion technique in the peripheral blood of 7 of the patients. Only patient S.C. was known to have received a blood transfusion (6 units) about 5 months prior to his illness. Patients T.S. and M.B. were technicians in the laboratory who had extensive contact with materials containing Australia antigen.

Lymphocytes from the peripheral blood were separated by the technique of Bach and Hirschhorn (12). Final concentration of cells was adjusted to 0.75×10^6 cells/ml. Lymphocytes were cultured in triplicate from each individual in the presence of phytohemagglutinin M (0.025 ml/ml of culture). Cultures from both members of the pair, *i.e.*, the patient with viral hepatitis and the corresponding age- and sex-matched control, were

[1] This work was supported by USPHS Grants CA-08069, CA-06551, CA-11524, CA-06927, and FR-05539 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania and the Stanley C. Dordick Foundation. Dr. S. S. Agarwal was a Fellow of the International Agency for Research on Cancer, World Health Organization. This work was presented in part at the Annual Meeting of the American Federation for Clinical Research, May 1970.

TABLE I. Response of Lymphocytes from Patients with Viral Hepatitis to Phytohemagglutinin (PHA) *In Vitro*.[a]

Name	DNA polymerase activity (mμmoles of dTM^{32}P/hr/0.1 ml)		Rate of thymidine uptake (cpm/0.1 ml)	
	Normal control	Patient	Normal control	Patient
J.S.	0.101	0.003	3844	291
T.S.	0.064	0.002	8392	142
B.Y.	0.064	0.024	8392	7208
L.H.	0.018	0.004	2647	965
S.C.	0.018	0.001	2647	45
M.B.	0.108	0.016	7829	2888
E.K.	0.057	0.048	1555	1822
G.W.	0.046	0.008	1821	170
C.W.	0.046	0.018	1821	192

[a] Lymphocyte cultures were incubated with PHA for 3 days. DNA polymerase activity and the rate of thymidine uptake were measured as described in "Methods." Results are given as the mean of the triplicate cultures. Cultures showing variation of over 10% from the mean were not included (only one in the above study).

cultured simultaneously under identical conditions. The cultures were harvested at 66 hr. Two hours prior to the termination of the cultures ^{3}H-thymidine (2.5 μCi/ml) was added to each culture. The cells were then washed and frozen and thawed two times. The cell lysate was used for the assay of DNA polymerase activity. (This assay measures the incorporation of deoxynucleoside triphosphates into an acid-insoluble product in the presence of added DNA which serves as template for the reaction.) For this purpose alpha-^{32}P-labeled thymidine triphosphate (TTP) was used as one of the substrates. The reaction was stopped by cold perchloric acid and the acid-insoluble precipitate was collected on glass fiber filters. The ^{3}H and ^{32}P counts were determined simultaneously by liquid scintillation counting. After correcting for crossover, tritium counts represent incorporation of thymidine into cellular DNA *in vivo* and ^{32}P counts represent DNA polymerase activity of the same set of cells. The details of the techniques have been previously described (8). The results were analyzed statistically by Wilcoxon's matched-pair signed-ranks test.

Results. The ability of phytohemagglutinin to stimulate DNA replication in cultures of human peripheral lymphocytes was determined by measuring the increase in DNA polymerase activity and the rate of thymidine incorporation. The lymphocytes from the patients with viral hepatitis were hyporesponsive; in all the 9 comparisons, values for the DNA polymerase activity were lower in patients with viral hepatitis than matched controls (Table I). Except for one patient (E.K.), the values for thymidine incorporation were similarly lower in PHA-stimulated lymphocytes from patients with viral hepatitis than in the controls. The p value by Wilcoxon's matched-pair signed-ranks test was <0.01 for both DNA polymerase activity and thymidine incorporation. There was no obvious relationship between the extent of stimulation with PHA and the duration of symptoms or the severity of the disease (Table II). In one patient (B.Y.) the uptake of thymidine was close to that of the control although the DNA polymerase activity was significantly lower. In another patient (E.K.), the value for thymidine incorporation was higher than that of the control, yet DNA polymerase activity was lower. These discrepancies in the two parameters of DNA synthesis are explainable since the rate of incorporation of radioactive exogenous thymidine may be influenced by the variations in extra- and intracellular concentration of nonradioactive metabolites of thymidine along the pathway of thymidine utilization. Thus, DNA polymerase activity could more accurately reflect the ability of the cells to synthesize DNA.

Patient M.B. had volunteered for a lym-

TABLE II. Summary of the Clinical and Laboratory Findings.[a]

Name	Day on which lymphocytes were cultured after the onset of: Symptoms	Jaundice	Serum bilirubin (total/direct; mg/100 ml)	SGOT (Karmen units)	SGPT (Karmen units)	Alkaline phosphatase (BU)[b]	Australia antigen [AU(1)]
J.S.	17	14	7.4/ 3.7	299	805	6.6	—
T.S.	70	60	8.4/ 4.5	243	1710	3.8	+
B.Y.[c]	—[d]	17	34.4/16.7	775	227	5.6	+
L.H.[e]	28	14	2.0/ 0.35	450	960	7.6	+
S.C.[f]	—[g]	16	25.2/10.6	568	1060	6.2	+
M.B.	8	4	6.2/ 3.4	180	3240	5.3	+
E.K.	14	8	12.5/ 5.2	450	312	9.9	+
G.W.	17	15	13.0/ 6.5	730	449	12.4	+
C.W.	17	13	6.5/ 2.7	1575	415	11.7	—

[a] Laboratory results are the highest values during the course of the disease.
[b] Bodansky units.
[c] Histological diagnosis of viral hepatitis at autopsy.
[d] Good history not available.
[e] Percutaneous liver biopsy showed evidence of persistent active hepatitis.
[f] Cholestatic hepatitis on needle biopsy.
[g] No preicteric symptoms.

phocyte stimulation study about a year prior to the attack of acute hepatitis. At that time her lymphocytes responded normally to PHA. The DNA polymerase activity was 0.108 mμmoles of dTM^{32}P/hr/0.1 ml. Four days after the appearance of jaundice, the DNA polymerase activity of the PHA-stimulated lymphocytes was 0.016 mμmoles of dTM^{32}P/hr/0.1 ml. On day 21 after the onset of illness, when she was clinically better, the response had significantly increased (see Table III). This finding suggests that the impaired responsiveness is transient and occurs only during the acute phase of hepatitis. In another patient (T.S.), hyporesponsiveness to PHA also disappeared after complete clinical recovery. In contrast, the lymphocytes from patient L.H., 2 weeks after the first study were still hyporesponsive to PHA. Clinically he was still sick and a percutaneous needle liver biopsy showed evidence of persistent active hepatitis.

TABLE III. Responsiveness of Lymphocytes to PHA During the Course of Disease.

Name	Date of study	Clinical status	DNA polymerase activity (mμmoles of dTM ^{32}P/hr/0.1 ml): Normal control	DNA polymerase activity (mμmoles of dTM ^{32}P/hr/0.1 ml): Patient	Rate of thymidine uptake (cpm/0.1 ml): Normal control	Rate of thymidine uptake (cpm/0.1 ml): Patient
M.B.	5/16/68	Prior to onset of illness	—	0.108	—	—
	4/22/69	Active disease	0.108	0.016	7829	2888
	5/16/69	Convalescence	0.057	0.055	1555	1549
T.S.	10/29/68	Posticteric phase	0.064	0.002	8392	142
	5/16/69	Complete recovery	0.057	0.164	1555	3413
L.H.[a]	11/8/68	Active disease	0.018	0.004	2647	965
	11/22/68	Active disease	0.063	0.005	7578	1592

[a] Percutaneous liver biopsy showed evidence of persistent active hepatitis.

In order to exclude the effect of any substance in the plasma of these patients on PHA responsiveness of the lymphocytes, the cells were washed carefully with Eagle's MEM before culturing. In the final cultures, the expected concentration of the patients' plasma would have been less than 1%. The data on the effect of plasma from patients with viral hepatitis on the responsiveness of normal lymphocytes is presented in the following paper.

Discussion. The present study provides quantitative evidence that DNA replication in PHA-stimulated lymphocytes from patients with viral hepatitis is significantly impaired compared with normal healthy subjects ($p<0.01$). There is not only an impairment of thymidine incorporation, but the overall ability of the cells to synthesize DNA, as measured by DNA polymerase activity, is also diminished. This impairment in responsiveness does not seem to be due to the presence of any substance in the plasma of these patients (see following paper) and is a transitory phenomenon seen only during the active phase of the disease. In two patients in this study (M.B. and T.S.) lymphocyte responsiveness returned to normal following recovery from the disease. However, in patient L.H., who entered into a phase of persistent active hepatitis, lymphocytes were still hyporesponsive after a period of 2 weeks. Additional studies are required to determine if the lymphocyte stimulation test could be of value in evaluating the course of hepatitis.

These results support the earlier observations of Mella and Lang (10), who reported that the lymphocytes from patients with infectious hepatitis show a poor mitotic response to PHA but they differ in details from those reported by Willems *et al.* (5) who measured thymidine incorporation. The latter did not find any difference in the peak rate of thymidine uptake in the PHA-stimulated lymphocytes from patients with infectious hepatitis compared to normals; but in 3-day cultures, the values were significantly lower in the lymphocytes from the patients. However, our results may not be directly comparable with theirs. In their system, maximum rate of thymidine incorporation in control cultures is attained after 6 days of incubation in the presence of PHA, while in our system both the maximum rate of thymidine incorporation and maximum increase in DNA polymerase activity in lymphocytes from normal volunteers is attained after 66 to 80 hr in culture.

In vitro lymphocyte stimulation test has been used to evaluate cellular immune status of individuals by several investigators (for references, see Oppenheim) (13). Impairment in responsiveness to mitogenic stimuli such as PHA in lymphocytes from patients with viral hepatitis and some other viral infections could indicate that viral infections interfere with cellular immune defense mechanisms. In fact, this has been shown in humans as well as in laboratory animals for several nononcogenic and oncogenic viruses (14). Interference with lymphoid cell proliferation could be important in the genesis of immune hyporesponsiveness in the presence of viral infections.

We have been systematically studying the DNA polymerase activity in PHA-stimulated lymphocytes in patients with diseases associated with Australia antigen. This antigen persists in Down's syndrome (9) chronic lymphocytic leukemia (15), and in lepromatous leprosy (16). We had earlier reported that lymphocytes from patients with Down's syndrome are hyporesponsive to PHA *in vitro* (8). Similar impairment has been reported for the lymphocytes from patients with chronic lymphatic leukemia (17, 18) and lepromatous leprosy (19) by other workers. We do not know if this apparent relationship between persistent Au(1) and lymphocyte hyporesponsiveness could result from either: (i) impaired immune responsiveness making these persons more susceptible to persistence of Australia antigen; or (ii) the presence of Australia antigen contributing to impairment in responsiveness to PHA. The results of the addition of purified Au(1) to PHA-stimulated lymphocytes from normal volunteers are presented in the following paper.

The exact mechanism of the action of PHA is still a subject of speculation. However, a number of other antigens, when added to

lymphocyte cultures, can similarly stimulate the DNA replication and blastogenesis. This is also true for the HL-A alloantigens (human transplantation antigens) which are responsible for the blastogenic response in the mixed leukocyte culture reaction (20). It is likely that the lymphocytes from patients with viral hepatitis may be hyporesponsive not only to PHA, but to other antigenic stimuli as well. A high prevalence of hepatitis often associated with Australia antigen has been reported from several chronic renal dialysis units (21). The hepatitis is often of long duration, anicteric, and not usually suspected clinically. Since these patients are potential graft recipients, mixed lymphocyte culture tests are often used for histocompatibility matching. We would suggest that the results of such tests should be interpreted with caution since a weak response could be the result of lymphocytes being hyporesponsive due to hepatitis rather than true tissue compatibility.

Summary. Lymphocytes from patients with viral hepatitis were found to be hyporesponsive to the replicating stimulus of phytohemagglutinin (PHA) *in vitro*. This was shown by an impairment both in the induction of DNA polymerase activity and thymidine incorporation into the cellular DNA by PHA-stimulated lymphocytes. The deficit is transient and occurs with the onset of clinical symptoms.

We acknowledge with appreciation the technical help of Sylvia J. Bugbee.

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Received Mar. 4, 1971. P.S.E.B.M., 1971, Vol. 137.