Lymphocyte Transformation and Hepatitis
II. Lack of Direct *in Vitro* Inhibition by Purified Australia Antigen[1] (35861)

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The ability of bacteriophage and animal viruses to interfere with host cell replication is well documented. The induction of DNA replication in human peripheral blood lymphocytes by phytohemagglutinin (PHA) can be inhibited by several animal viruses (1–3). Lymphocytes from patients with viral hepatitis are hyporesponsive to PHA.[3] In the preceding paper this hyporesponsiveness was shown to be associated with a deficit in the induction of DNA polymerase and a decrease in the ability of lymphocytes to incorporate thymidine into DNA. The hyporesponsiveness was present during acute hepatitis and returned to normal before recovery. In one chronic case the cells remained hyporesponsive during the course of the disease. The hyporesponsiveness of the lymphocytes from patients with viral hepatitis could be due to the presence of a factor in the cells or in the serum which inhibits the cell response to PHA. In these studies we attempted to determine whether Australia antigen [Au(1)] interferes with DNA replication during lymphocyte transformation *in vitro*. We measured the effects of plasma and frozen–thawed lymphocytes from patients with viral hepatitis, and of purified Au(1) on the PHA stimulation of DNA replication in normal lymphocytes. We found that plasma, lymphocytes, and purified Au(1) do not affect the induction of DNA polymerase activity and the ability of the cells to incorporate thymidine into DNA during lymphocyte transformation.

Materials and Methods. 1. Plasma from patients with acute viral hepatitis. Whole blood was collected in heparinized vacutainer tubes from 5 patients with viral hepatitis. The time of obtaining plasma varied between 4 to 60 days from the onset of jaundice. The plasma from patient L.H. was obtained on day 14. Australia antigen was detected in the peripheral blood of 3 of the 5 patients (B.Y., L.H., and S.C.) at the time blood was drawn. The tubes were centrifuged at 2000 rpm; and the plasma was separated. The diagnosis of hepatitis was made clinically and on the basis of liver function tests. Plasma was heated at 56° for 30 min to inactivate complement prior to use in each experiment.[4]

2. Frozen–thawed lymphocytes from patients with viral hepatitis. Lymphocytes from the peripheral blood of three patients with viral hepatitis were collected according to the method of Bach and Hirschhorn (4). Cell concentration was adjusted to 0.75×10^6 cells/ml of culture medium. The lymphocytes were rapidly frozen and thawed two times prior to adding them to cultures of lymphocytes from normal human volunteers.

3. Purified Au(1) fractions. The Au(1) was purified by the method described earlier (5) from 2.0 ml of serum from the following sources:

a. Two patients (M.B. and J.C.) with acute viral hepatitis.

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[3] Preceding paper [Proc. Soc. Exp. Biol. Med. 137, 1498 (1971)].

[4] Au(1) is resistant to this treatment as determined by immunodiffusion reactivity and appearance under the electron microscope (5).

TABLE I. Effect of Plasma and Frozen–Thawed Lymphocytes from Patients with Viral Hepatitis on the Response of Lymphocytes from Normal Healthy Volunteers to Phytohemagglutinin Stimulation.

Expt. no.[a]	DNA polymerase activity (d-TM^{32}P/hr/0.1 ml)			Thymidine uptake (cpm/0.1 ml)		
	Without any addition	With plasma from patient	p	Without any addition	With plasma from patient	p
1. (T. S.)	0.064 ± 0.023	0.093 ± 0.027	>.05	8392 ± 1164	10,909 ± 602[b]	>.05
2. (B. Y.)	0.064 ± 0.023	0.074 ± 0.024	>.05	8392 ± 1164	7091 ± 252[b]	>.05
3. (L. H.)	0.018 ± 0.005	0.068 ± 0.007[c]	>.01	2647 ± 401	6062 ± 521	>.01
4. (S. C.)	0.031 ± 0.014	0.008 ± 0.008[d]	>.05	3759 ± 1122	1243 ± 696	>.05
5. (M. R.)	0.108 ± 0.014	0.082 ± 0.020[d]	>.05	7829 ± 1471	4699 ± 1099	>.05
		With frozen–thawed lymphocytes from patient			With frozen–thawed lymphocytes from patient	
1. (L. H.)	0.063 ± 0.022	0.052 ± 0.017	>.05	7578 ± 3317	5121 ± 361	>.05
2. (S. C.)	0.031 ± 0.014	0.021 ± 0.003	>.05	3759 ± 1122	1246 ± 166	>.05
3. (M. B.)	0.108 ± 0.014	0.080 ± 0.013[e]	>.05	7829 ± 1471	5462 ± 1027	>.05

[a] Each experiment was performed with plasma and frozen–thawed lymphocytes from different patients with viral hepatitis: Plasma was "heat inactivated" at 56° for 30 min; 0.5 ml of the plasma was added to 2-ml cultures; results are given as mean ± SD.

[b] Heat-inactivated plasma from a patient with viral hepatitis by itself did not have any stimulatory effect on normal human lymphocytes. DNA polymerase activity was 0.002 and thymidine uptake was 264 in Expt. 1, and the corresponding values in Expt. 2 were 0.002 and 215, respectively.

[c] In one culture, the value for DNA polymerase activity was 0.008 and thymidine uptake was 1367.

[d] Equivalent amount of heat-inactivated plasma from a normal volunteer did not have any effect. The DNA polymerase activity was 0.035 ± 0.007 and the thymidine uptake was 4143 ± 567 in Expt. 4, and the corresponding values for Expt. 5 were 0.094 ± 0.004 and 6258 ± 202, respectively.

[e] On the addition of frozen–thawed lymphocytes from a normal person, the DNA polymerase activity was 0.050, and the thymidine uptake was 2124 in one culture.

b. One patient (C36346 Cebu, Philippines) with lepromatous leprosy.

c. One patient (T.M.) with Down's syndrome and chronic anicteric hepatitis. In brief, the isolation procedure included enzymatic treatment to degrade normal serum proteins, passage through Sephadex G 200, and centrifugation on sucrose and cesium chloride gradients. Four sera from normal individuals were purified simultaneously in the same manner as the sera containing Au(1) to serve as controls. The cesium chloride density gradient fractions containing purified Au(1) and comparable fractions of control sera were dialyzed and the nondialyzable portion was tested for its effect on PHA stimulation of normal lymphocytes.

4. Culture of lymphocytes from normal healthy volunteers. Lymphocytes from the peripheral blood of 10 individuals were cultured in triplicate according to the method described previously (6). At the time the culture was started, 0.25 ml of PHA (M) was added to each milliliter of cell suspension containing 0.75 × 10^6 cells. (i) Plasma containing Au(1), or (ii) frozen–thawed lymphocytes from blood containing Au(1), or (iii) purified Au(1) were added at the same time as PHA to the lymphocyte cultures. After incubation for 66 hr at 37°, the rate of incorporation of ^{3}H-thymidine, and DNA polymerase activity were determined (6).

The other methods used are the same as those reported in the previous paper.[3]

TABLE II. Effect of Purified Au(1) and Control Fractions on the Response of Lymphocytes from Five Normal Healthy Volunteers (A to E) to Phytohemagglutinin Stimulation—DNA Polymerase Activity (mμmoles/hr/culture ± 1 SD).[a]

		PHA	PHA + Au(1)			PHA + Au(0)		
			I	II	III	IV	V	VI
A		0.075 ± 0.005	0.056 ± 0.004	0.057 ± 0.01	0.053 ± 0.013	0.051 ± 0.02	0.053 ± 0.000	0.066 ± 0.004
	p		<.05 >.01	>.05	>.05	>.05	<.01	>.05
B		0.551 ± 0.008	0.522 ± 0.034	0.532 ± 0.026	0.518 ± 0.006	0.506 ± 0.001	0.515 ± 0.021	0.488 ± 0.051
	p		>.05	>.05	<.05 >.01	<.01	>.05	>.05
C		0.349 ± 0.021	0.314 ± 0.008	0.348 ± 0.02	0.315 ± 0.008	0.296 ± 0.019	0.287 ± 0.018	0.263 ± 0.001
	p		>.05	>.05	>.05	>.05	>.05	<.05 >.01
D		0.159 ± 0.026	0.140 ± 0.005	0.156 ± 0.005	0.143 ± 0.007	0.166 ± 0.003	0.130 ± 0.003	0.153 ± 0.010
	p		>.05	>.05	>.05	>.05	>.05	>.05
E		0.253 ± 0.008	0.400[b]	0.415 ± 0.023	0.370[b]	0.372 ± 0.014	0.380 ± 0.065	0.399 ± 0.020
	p			<.01		<.01	>.05	<.01

[a] I, Au(1) isolated from a lepromatous leprosy patient from Cebu, Philippines; II, M.B. (acute hepatitis); III, T.M. (Down's syndrome); normal serum fraction: IV, no. 667; V, no. 790; and VI, no. 665.

[b] Result of only 1 culture available.

Results. Effect of plasma from patients with viral hepatitis on lymphocyte responsiveness to PHA. In 4 of 5 experiments (Table I) there was no significant effect on the extent of PHA stimulation of normal lymphocytes as measured by DNA polymerase activity and ^{3}H-thymidine incorporation ($p > 0.05$). The results of DNA polymerase activity and thymidine incorporation are in good agreement. In one experiment (L.H.) there was greater stimulation of lymphocytes by PHA in the presence of plasma from the patient with hepatitis. However, the plasma from this patient and also the plasma from two other patients had no stimulatory effect on the lymphocytes by itself (*i.e.*, without PHA). An equivalent amount of heat inactivated plasma from two normal healthy volunteers had no effect on PHA stimulation of lymphocytes.

Effect of frozen–thawed lymphocytes from patients with viral hepatitis on the responsiveness of normal lymphocytes to PHA. In the previous paper it was reported that lymphocytes from patients with viral hepatitis are hyporesponsive to PHA even when well-washed lymphocytes were cultured in heterologous serum. This suggests that lymphocytes by themselves are hyporesponsive. In order to determine whether lymphocytes from patients with viral hepatitis contain inhibitory substances, frozen–thawed lymphocytes from 3 patients with viral hepatitis were added to cultures of normal lymphocytes stimulated with PHA. The results are given in Table I. Although absolute values suggest an inhibitory effect the differences are not significant statistically.

Effect of purified Au(1) on the PHA responsiveness of lymphocytes from normal individuals. The experiments described above did not identify the substances in the blood of patients with hepatitis which result in the hyporesponsiveness of lymphocytes from normal patients. Since we are testing the hypothesis that Au(1) is related to the hyporesponsive reaction we next studied the effect of purified Au(1) on the lymphocytes from normal individuals. The results of the first experiment are given in Table II. The initiation of DNA replication was determined by

TABLE III. Effect of Purified Au(1) and Control Fractions on the Response of Lymphocytes from Five Normal Healthy Volunteers (A to E) to Phytohemagglutinin Stimulation—Rate of ^{3}H-Thymidine Incorporation (cpm/hr/culture ± 1 SD).[a]

	PHA	PHA + Au(1)			PHA + Au(0)		
		I	II	III	IV	V	VI
A	3959 ± 463	3255 ± 214	3744 ± 75	3346 ± 355	3351 ± 594	2773 ± 461	3571 ± 143
p		>.05	>.05	>.05	>.05	>.05	>.05
B	30,662 ±1242	28,290 ±1163	26,563 ±1511	25,948 ±1630	25,767 ±1870	27,294 ± 873	21,856 ±3151
p		>.05	>.05	<.05 >.01	>.05	>.05	<.05 >.01
C	16,628 ± 441	14,045 ± 810	19,750 ± 191	19,265 ±1051	17,730 ±1276	15,508 ±1296	16,650 ± 909
p		<.05 >.01	<.01	>.05	>.05	>.05	>.05
D	7152 ± 272	5740 ± 707	5821 ± 242	5421 ± 306	6381 ± 163	5215 ± 76	6199 ± 397
p		>.05	<.05 >.01	<.01	>.05	<.01	>.05
E	10,665 ± 328	7446	8201 ± 194	6496	6992 ± 591	7165 ±1153	7604 ± 263
p			<.01		<.01	>.05	<.01

[a] Same footnotes as to Table II.

TABLE IV. Effect of Purified Au(1) from Acute Hepatitis Serum on the Response of Lymphocytes from Normal Healthy Volunteers to PHA Stimulation.

Normal lymphocytes		DNA polymerase				
		PHA	PHA			
			+ Au(1)[a]	+ Au(1)[b]	+ $CsCl_2$ cont.[c]	+ $CsCl_2$ pellet[d]
A		0.288 ± 0.035	0.210 ± 0.021	0.208 ± 0.013	0.194 ± 0.015	0.170 ± 0.013
	p		>.05	>.05	>.05	<.05 >.01
B		0.294 ± 0.015	0.263 ± 0.027	0.244 ± 0.013	0.239 ± 0.017	
	p		>.05	>.05	>.05	
C		0.327 ± 0.028	0.315 ± 0.004	0.304 ± 0.001	0.301 ± 0.026	
	p		>.05	>.05	>.05	
D		0.405 ± 0.036	0.382 ± 0.025	0.359 ± 0.025	0.332 ± 0.032	0.373 ± 0.026
	p		>.05	>.05	>.05	>.05

[a] Purified Au(1) processed as previously reported (5).

[b] Purified Au(1) processed as previously reported (5) but reversing the order of $CsCl_2$ and sucrose gradient fractionation. The Au(1) used here represents the active pool of sucrose gradient fractions.

[c] $CsCl_2$ (density 1.3) dialyzed versus 0.01 *M* KCl. The nondialyzable fluid was tested here.

[d] The precipitate remaining in $CsCl_2$ gradient tube. It did not produce a precipitin band by immunodiffusion.

measurements of DNA polymerase activity. Purified Au(1) was added to PHA-stimulated lymphocyte cultures from normal individuals; as shown, there was a small decrease in the mean response of lymphocytes to PHA in the presence of Au(1). However, a similar decrease was observed when control fractions (*i.e.*, those containing no Australia antigen) were added to the culture. With one culture (E, Table II) there was a significant stimulation of PHA response when Au(1) was added, but a similar response was also observed in those cultures to which control fractions were added. Thus, this effect can not be specifically attributed to Au(1). Similar results were obtained in measurements of thymidine incorporation (Table III).

In the above experiment only one of the three sources of purified Australia antigen (M. B.) was a patient with acute viral hepatitis. In the final purified preparation from this patient, Au(1) was not detectable by the immunodiffusion technique and thus may not have been present. Therefore Au(1) was isolated and purified from another patient (J. C.) with acute viral hepatitis and its effect on the PHA responsiveness of lymphocytes from normal individuals was studied. Fractions containing Au(1) (by the immunodiffusion technique) from both the cesium chloride and sucrose density gradients were also tested. In addition, the effect of the pellet from the cesium chloride gradient was tested, since particles with a large amount of nucleic acid would have sedimented to the bottom of the tube during centrifugation. The results are given in Table IV. Again there is no significant depression of PHA responsiveness of the lymphocytes from normal healthy volunteers.

Discussion. Experimental inoculation of PHA-stimulated lymphocyte cultures with live rubella virus (7), polio virus (8), and some other viruses has resulted in impairment of DNA and RNA synthesis in both the abortive and the propagatable infections. While it is possible that the hyporesponsiveness of the lymphocytes from patients with viral hepatitis could be directly related to the presence of a virus in these cells, we have not been able to demonstrate an effect of the addition of either frozen and thawed lymphocytes or purified fractions of Australia antigen on normal lymphocytes stimulated with PHA. In infectious mononucleosis and congenital rubella, *in vitro* responsiveness to

the lymphocytes has been found to be impaired; and viruses, presumed to be the causative agents, have been successfully isolated from the lymphocytes (7, 8). In an earlier study, we did not find Australia antigen by fluorescent antibody technique in the peripheral blood lymphocytes from patients who had Australia antigen in their blood (10).

We currently favor as an interpretation of the present data that either lymphocytes do not provide a suitable system for the replication of Au(1) *in vitro* or that hyporesponsiveness of the lymphocytes to PHA from the patients with acute viral hepatitis is not directly due to an effect of Australia antigen.

Summary. Plasma, frozen and thawed lymphocytes, and purified Australia antigen fractions from various patients suffering from viral hepatitis were tested for an effect on PHA-stimulated lymphocytes isolated from normal individuals. The addition of these materials to lymphocyte cultures produced no significant change from that of PHA alone or PHA plus control materials from normal (nonhepatitis) patients.

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