Viral Inhibition of the Phytohemagglutinin Response of Human Lymphocytes and Application to Viral Hepatitis* (33628)

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Several viruses have been demonstrated to multiply in human leukocytes cultured *in vitro* (1–6). Some require the addition of a mitogenic substance such as phytohemagglutinin (PHA) (2, 6, 7), while for others the mitogenic substance is not essential, but does enhance viral replication in the leukocyte cultures. Lymphocytes obtained soon after birth from infants with congenital rubella are usually unresponsive to the mitogenic stimulation of PHA (8–10). *In vitro* rubella virus inhibits the blastogenic response of normal lymphocytes to several mitogens (9, 10). The purpose of the present study was to determine whether the effect of viruses on the mitogenic response of lymphocytes might provide a method of detecting and assaying viruses which do not produce cytopathic changes in tissue culture systems. In addition to investigating a number of known viruses, we attempted to detect a transmissible agent in serum or leukocytes from patients with viral hepatitis, Hodgkin's disease, and chronic lymphatic leukemia.

Materials and methods. Viruses. Primary African green monkey kidney (GMK) cells were used to prepare stocks of poliovirus type 1 (Mahoney strain), echovirus 11 (Gregory strain), mumps virus (a fresh laboratory isolate), vesicular stomatitis virus (VSV) (Indiana serotype) and reovirus type 3 (CAN 246 strain). A stock of simian virus 40 (SV40) (Baylor strain) (11) was prepared in CV-1 cells. Stocks of influenza A (PR/8 strain), influenza A_2 (Japan 305 strain), influenza B (Lee strain), Newcastle disease virus (NDV) (California strain), Sendai virus (52 strain) and Sindbis virus (Ar 339 strain) were prepared in the chorioallantoic cavity of 11 day embryonated eggs. Herpes simplex virus (KOS strain) and vaccinia virus (WR strain) stocks were prepared in primary rabbit kidney cells. Stocks of human adenovirus type 2 (a fresh isolate), type 7 (LL strain) and type 12 (Huie strain), and their satellite viruses (types 1–3) were prepared in human embryonic kidney cells. Wart virus was obtained by making a 10% extract in distilled water of excised human specimens. The virus stocks were tested and found to be free of mycoplasma.

Virus assays. Poliovirus. VSV, vaccinia virus and reovirus were assayed by the plaque counting method on BSC-1 cells using a single agar overlay. Sindbis virus and NDV were assayed similarly using primary chick embryo fibroblast; the adenoviruses were plaqued in human embryo kidney cells. The SV40 virus was assayed by the plaque counting method using GMK cells. Herpes simplex virus was assayed by the plaque counting method using primary rabbit kidney cells and an overlay containing methylcellulose. Echovirus 11 was assayed by cytopathic effect in GMK cells. Wart virus and adeno-associated satellite viruses were assayed by the particle count method in the electron microscope. The remaining viruses were assayed in GMK cells by the hemadsorption technique.

Culture media. Tissue culture cells used for preparing virus stocks and for assay were

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grown in Melnick's medium A or Eagle's medium supplemented with fetal calf serum, 10%; sodium bicarbonate, 0.75 g/liter; penicillin, 100 units/ml; and streptomycin, 100 μg/ml. Maintenance medium consisted of Eagle's medium supplemented with fetal calf serum, 2%; sodium bicarbonate, 1.50 g/liter; and antibiotics. Agar overlay medium for the plaque counting method contained Bacto agar, Eagle's medium, fetal calf serum, 10%; sodium bicarbonate, 2.25 g/liter; neutral red, and antibiotics. For assay of herpes virus, the agar in the overlay was substituted with methocel 2% (Fisher Scientific). Plaquing was performed in 60-mm plastic petri dishes and the cultures were incubated in 5% CO_2 in air. Leukocytes were cultured in Eagle's medium containing twice normal concentration of glutamine, heat inactivated fetal calf serum, 20%; sodium bicarbonate, 2.25 g/liter; penicillin; and streptomycin.

Leukocyte cultures. Leukocyte cultures from patients with viral hepatitis, Hodgkin's disease, or chronic lymphatic leukemia and from healthy donors were prepared as described (9). The cultures contained approximately 1×10^6 leukocytes in 2 ml. Phytohemagglutinin (PHA)-M (Difco, lot 510957) was diluted 1:50 in saline and 0.1 ml of this dilution was added to each culture. Cultures were incubated at 35° in a humidified atmosphere of 5% CO_2 in air. At least three cultures for each virus specimen were harvested after 6 days incubation, unless otherwise indicated. After a 5-hr labeling period with 0.2 ml of thymidine-^{3}H (1 μCi; sp act. 2 Ci/mmole), leukocytes were sedimented by centrifugation at 1000 rpm for 15 min and washed once in cold Hanks' BSS. Cold trichloracetic acid (5%) was added and the acid-insoluble precipitate was sedimented at 1800 rpm for 20 min. The sediment was dissolved in 1 ml of 0.1 *M* sodium hydroxide and again precipitated with 4.5 ml of cold trichloracetic acid 6.7%. The final precipitate was dissolved in 0.1 ml of hyamine 10-X (Packard Corp.), mixed with scintillation fluid and counted in a Beckman liquid scintillation counter. Cultures not labelled with thymidine-^{3}H served for viability tests, which were performed by means of the dye exclusion method with trypan blue, and for cell counts which were performed in a hemocytometer. Additional cultures were frozen and thawed once and used for assay of virus yield and passage to fresh leukocyte cultures.

Donors for leukocyte cultures. Blood samples were taken from healthy donors selected from our laboratory personnel. Leukocyte cultures from these samples were used for replication studies with the known viruses and served as controls for other studies. Fifteen patients with clinically diagnosed viral hepatitis were investigated. The patients were admitted to the hospital with moderate to severe jaundice. Their sera contained elevated bilirubin, glutamic-oxalacetic transaminase, and glutamic-pyruvic transaminase values. Liver biopsy performed on some of these patients strongly supported the diagnosis of viral hepatitis. Six hospitalized patients with diseases other than viral hepatitis also served as controls. Blood samples were taken from 4 patients with Hodgkin's disease and from 2 patients with chronic lymphatic leukemia; these patients were not receiving treatment at the time that the samples were taken for leukocyte cultures.

Results. Dose–response curve for PHA in leukocyte cultures. Because of variation in response of human lymphocytes to phytohemagglutinin *in vitro* (12), a dose–response curve of PHA was done in leukocyte cultures from 5 donors. Cultures were stimulated with PHA when the cultures were prepared (day 0) and harvested on day 6 after a 5-hr pulse with thymidine-^{3}H. Results as expressed in counts per minute (cpm) are shown in Fig. 1. A variation in absolute response for leukocyte cultures between the individuals is obvious. A maximum response was observed in 4 of the 5 preparations at a PHA-M concentration of 0.01 ml/culture, which was chosen for further experiments. The pattern of DNA synthesis, as detemined by uptake of thymidine-^{3}H, was examined daily starting on day 3 using a PHA concentration of 0.01 ml/culture. The results of this experiment are shown in Fig. 2. A peak of DNA synthesis is demonstrated on day 6,

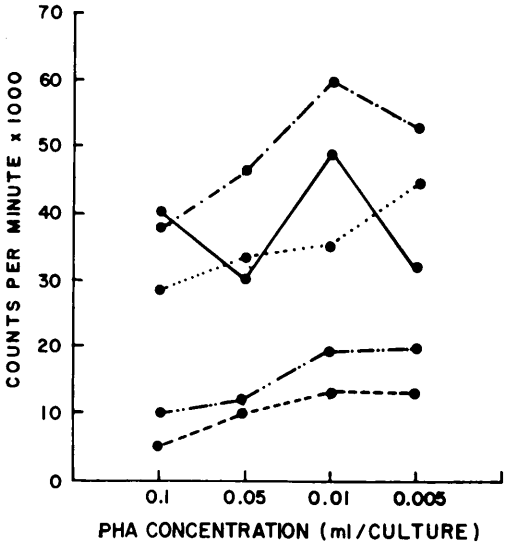

FIG. 1. The effect of PHA concentration on the DNA synthesis of leukocytes from 5 separate donors.

which agrees with the results of Moorhead and co-workers (13). Because of dying polymorphonuclear cells, the total number of leukocytes in the cultures drops until day 3 and then gradually increases until day 6. Some cultures reached the same number of cells or even more than originally present in the cultures. Cells on day 6 proved to be at least 80–90% "blast cells" when stained smears were examined. Leukocyte cultures not stimulated with PHA showed minimal DNA synthesis and the numbers of spontaneous "blast cells" were always less than 10%. In the unstimulated cultures, total number of cells dropped until day 6, and the viability in these cultures was approximately 50%. Data obtained from triplicate cultures of leukocytes from 67 donors were used to determine the variability of the test; values exceeding ± 40% were significant at the 95% confidence limits.

The inhibition of the lymphocyte response to PHA by RNA viruses. The effect on the blastogenic response of lymphocytes to PHA of representative RNA viruses belonging to the picornavirus, myxovirus, paramyxovirus, arbovirus, rhabdovirus, and reovirus groups was examined. In addition, efforts were made to determine if viral replication was necessary to produce the inhibitory effect. Cultures were inoculated with 0.2 ml of the undiluted virus suspension and the cultures were harvested after 6-days incubation according to the procedures described in "Materials and Methods." Then 0.2 ml of the harvest was passed to a series of fresh cultures from another donor. Two to three serial passages were made, and virus yield and DNA synthesis of the cells determined at each passage. Poliovirus type 1 and echovirus 11 inhibited PHA stimulation of the leukocyte cultures, and the effect could be passed to fresh cultures (Table I). These 2 viruses replicated in the leukocytes, a finding which confirms observations reported by others (14, 15). The decreased DNA synthesis in the infected cultures could not be accounted for by decreased cell survival. The mean DNA synthesis of infected cultures in 15 experiments with poliovirus was 71% less than in control cultures while mean viable cells in the infected cultures were 38% less than in control cultures. Vesicular stomatitis virus inhibited the incorporation of thymidine-^{3}H and replicated well in the leukocytes; however, the virus was cytopathic to the cells (3). Reovirus type 3 was also cytotoxic and the inhibition of DNA synthesis could be serially transferred although viral replication was not demonstrated by the method employed. Mumps virus replicated in the stimulated leukocyte cultures without producing a

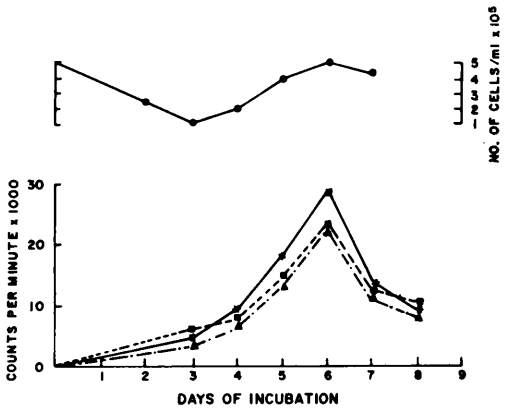

FIG. 2. The DNA synthesis and cell survival in leukocyte cultures containing 0.01 ml of PHA/culture.

TABLE I. Inhibition of PHA Response of Lymphocytes by RNA Viruses on Serial Passages.

Virus	Input	Inhibition (%) initial	Virus yield	Inhibition (%) passage 1	Virus yield	Inhibition (%) passage 2	Virus yield	Inhibition (%) passage 3	Virus yield
Poliovirus type 1	8.5×10^{7a}	—92[d]	6.7×10^{5}	—45	6.9×10^{5}	—70	8×10^{5}	—95	4.5×10^{5}
Echovirus type 11	$10^{7.5b}$	—83	$10^{5.6}$	—16	$10^{6.5}$	—65	$10^{5.7}$	—93	$10^{5.8}$
Influenza A/PR8	10^{7c}	—94	10^{4}	—23	10^{2}	—11	<10	NT[e]	NT
Influenza A_2/Jap.	$10^{6.5c}$	+ 3	10^{4}	0	$10^{2.5}$	—17	<10	NT	NT
Influenza B/Lee	$10^{6.5c}$	—56	10^{6}	—23	10^{3}	0	<10	NT	NT
Sendai	$10^{7.5c}$	—95	$10^{2.5}$	—16	<10	0	<10	NT	NT
Newcastle disease virus	2.5×10^{6a}	—50	1.5×10^{4}	—27	2.1×10^{2}	—20	1×10^{2}	0	<10
Mumps	$10^{5.5c}$	+ 2	$10^{4.5}$	—32	$10^{3.6}$	—20	$10^{3.8}$	—40	$10^{3.5}$
Sindbis	3.5×10^{6a}	+13	2.5×10^{3}	+13	3×10^{3}	0	1×10^{2}	+24	<10
Vesicular stomatitis virus	3×10^{7a}	—98	5×10^{4}	—67	3×10^{4}	—99	5×10^{4}	—99	4.5×10^{4}
Reovirus type 3	1.6×10^{7a}	—93	3×10^{4}	—70	2.1×10^{2}	—95	NT	NT	NT
Control PHA	—	0	—	+ 8	—	—13	—	+ 4	—

[a] Plaque forming units/ml.
[b] $TCID_{50}$/ml.
[c] $Hemadsorption_{50}$/ml.
[d] Infected as compared with control [1—(DNA synthesis with virus/DNA synthesis in control)] × 100.
[e] Not tested.

marked decrease in DNA synthesis or cytotoxicity (2). Influenza A, influenza B, Sendai and Newcastle disease viruses suppressed the response of the lymphocytes to PHA when the viruses were initially added at a high multiplicity of infection; however, these viruses appeared to replicate poorly and the inhibition of the DNA synthesis by the viruses could not be serially passaged. Control tissue culture fluids, chorioallantoic fluids, and serial passage materials from uninfected frozen and thawed leukocyte cultures failed to produce a suppression of the blastogenic response of the lymphocytes.

The inhibition of the lymphocyte response to PHA by DNA viruses. The results of similar experiments using DNA viruses are shown in Table II. Dramatic inhibition of DNA synthesis was observed in cultures initially infected with herpes simplex virus and vaccinia virus; however, only vaccinia virus replicated sufficiently for the effect to be serially passaged. As reported by Miller and Enders (6), cell survival in vaccinia-infected cultures was reduced, accounting for the decreased DNA synthesis. Similarly, decreased cell survival in herpesvirus-infected cultures was proportional to decreased thymidine-^{3}H incorporation. Slight inhibition of the PHA response of the lymphocytes was also observed in cultures initially infected with adenovirus type 12 and with the 3 types of adeno-associated satellite virus. Virus was recovered from cultures infected with SV40 and the human adenoviruses; however, the virus recovered on serial passage may represent surviving inoculum and not newly synthesized virus (16).

The PHA response of the lymphocytes was suppressed by the addition of wart virus particles. The mean inhibition of 15 experiments using 10–200 virus particles/cell was 40% while extracts of warts treated similarly, but not containing virus particles, suppressed incorporation of thymidine-^{3}H by only 7%. No evidence of replication of wart virus could be detected by electron microscopic examination of 6 day harvest material, and the inhibition of the blastogenic response of the lymphocytes was not present on serial passage (Table II).

Evaluation of the inhibition of the PHA response of lymphocytes in diseases of suspected viral etiology. To determine the lymphocyte response in viral hepatitis, the responsiveness to PHA of lymphocytes from patients with hepatitis was measured. Also the inhibitory effect of serum from hepatitis patients on lymphocytes from normal donors was studied. Leukocyte cultures from patients with serum and infectious hepatitis, from patients with diseases other than viral hepatitis and from healthy donors were prepared as described in "Materials and Methods." The cultures were harvested after 3 and 6 days of incubation. The DNA synthesis of the cells after 3 days of incubation was significantly less in leukocyte cultures from hepatitis patients than from patients with other diseases or from healthy donors (Fig. 3). This was observed in all leukocytes obtained from patients during the first 6 days after the onset of jaundice. Leukocytes collected later in the course of the illness did not regularly show this effect. Statistical analysis using the Student t test revealed the hyporesponsiveness of the cells collected during early illness to be significantly different from the controls ($p = < .01$). The DNA synthesis of the leukocytes from patients and healthy donors was essentially the same after 6 days of incubation. Sera from the donors with viral hepatitis were tested for the presence of the SH antigen associated with serum hepatitis (17). There was no correlation between the presence of the antigen and the hyporesponsiveness of the lymphocytes after 3 days of incubation.

Evidence of viral activity was also sought by determining the degree of inhibition of the PHA response of lymphocytes from normal donors by adding 0.2 ml of undiluted acute phase hepatitis serum to the leukocyte cultures and harvesting for analysis of DNA synthesis after 6 days of incubation. The mean inhibition of DNA synthesis produced by hepatitis sera in 10 experiments was 69% while sera from healthy donors in 8 experiments resulted in a mean inhibition of 56%.

TABLE II. Inhibition of PHA Response of Lymphocytes by DNA Viruses on Serial Passages.

Virus	Input[a]	Inhibition (%) initial	Virus yield	Inhibition (%) passage 1	Virus yield	Inhibition (%) passage 2	Virus yield	Inhibition (%) passage 3	Virus yield
Herpes type 1	6×10^7	−81	1.8×10^4	−38	<10	+ 8	<10	− 2	<10
Vaccinia	6×10^8	−97	1.2×10^5	−83	4.5×10^4	−80	NT	−91	NT
SV40	5.2×10^7	+33	1.8×10^6	−14	9.0×10^4	+10	5.6×10^4	+33	9.0×10^2
Adeno 2	8.5×10^8	+25	2.8×10^6	− 7	1.9×10^6	+22	2.1×10^4	+16	NT
Adeno 7	6.0×10^7	− 3	1.3×10^6	+25	2.2×10^4	+15	$<5 \times 10$	+ 7	NT
Adeno 12	8.5×10^6	−55	5.7×10^6	− 5	1.4×10^5	+18	5.0×10^1	NT	NT
AASV—Type 1	1×10^9	−51		0		+14		−14	
Type 2	2.5×10^9	−38		+ 4		+28		−18	
Type 3	1.3×10^9	−51		− 4		+ 6		−14	
AASV + AD7—Type 1	1×10^9	−16		+38		−28		− 1	
Type 2	2.5×10^9	−37		+ 4		0		−19	
Type 3	1.3×10^9	− 2		+ 1		+18		−18	
Human warts—Prep 1	1×10^9	−58		− 2		+ 1		− 5	
Prep 2	1×10^9	−44		−11		NT		NT	
Prep 3	1×10^9	−14		− 6		+28		+ 3	

[a] Input for herpes, vaccinia, SV40, and adenoviruses is given in plaque-forming units/ ml; input for AASV, AASV + AD7, and human warts is given in particles/ml.

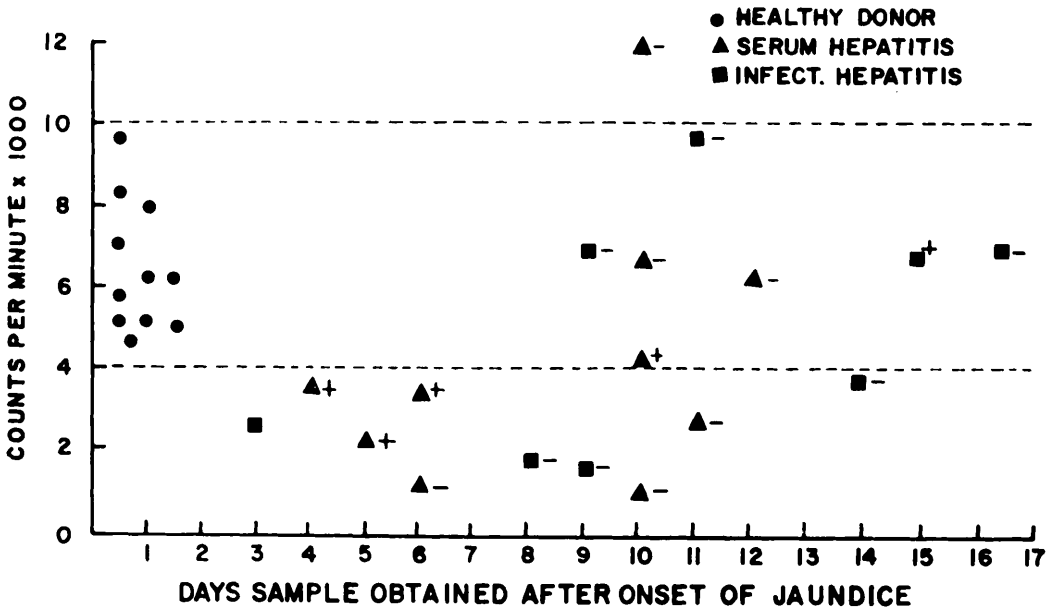

FIG. 3. The response to PHA of leukocytes from hepatitis patients and healthy donors; DNA synthesis after 3 days of incubation; (+) indicates the presence of SH antigen in the patient's serum.

Normal serum inhibits the PHA response of lymphocytes (18) and the difference between the inhibition by the hepatitis sera and control sera was not significant.

Cultures which yielded an inhibition of DNA synthesis produced by adding serum from hepatitis patients to cells from normal donors were tested for the presence of a serially transmissible agent. SH antigen-containing sera from 2 patients with serum hepatitis, 2 sera from patients with infectious hepatitis, and sera from 2 healthy donors were added to leukocyte cultures from healthy donors. The cultures were incubated for 6 days, and the DNA synthesis was determined in half of the cultures while the remaining cultures were frozen, thawed, and an aliquot was transferred to fresh leukocyte preparations. The results, shown in Table III, demonstrate that the inhibition of responsiveness to PHA following the initial addition of sera could not be serially transmitted.

Hodgkin's disease and chronic lymphocytic leukemia are two acquired diseases which are associated with an impaired response of the lymphocytes to PHA (19). Experiments designed to detect a transmissible agent which would confer the unresponsiveness to normal lymphocytes consisted of incubating

TABLE III. PHA Response of Leukocyte Cultures Inoculated with Viral Hepatitis Sera and Effect of Serial Passages.

Specimen	Specimen no.	Inhibition (%) initial	Inhibition (%) passage 1	Inhibition (%) passage 2	Inhibition (%) passage 3
Serum hepatitis	1	−68	+11	−14	+ 8
	2	−85	−10	+ 5	NT
Infectious hepatitis	1	−83	+16	− 7	NT
	2	−79	+ 7	− 6	+13
Serum control	1	−11	+10	−15	+ 4
	2	−14	− 5	+ 8	NT

TABLE IV. PHA Response of Lymphocytes from Patients with Hodgkin's Disease and Chronic Lymphocytic Leukemia and from Healthy Donors.

Expt.	Donor	Initial response	Passage
1	Hodgkin	711[a]	27,400
	Control	53,915	36,586
2	Hodgkin	1220	26,168
	Hodgkin	1389	21,979
	Control	26,047	25,851
3	Chronic lymphocytic leukemia	8705	61,024
	Control	27,102	31,852
	Control	59,292	54,391
4	Chronic lymphocytic leukemia	106	91,635
	Control	11,977	48,630
5	Hodgkin	4691	36,613
	Control	42,448	39,120

[a] Counts per minute.

leukocytes from patients and from healthy donors for 6 days in the presence and absence of PHA. Aliquots of frozen and thawed cultures were then used to inoculate fresh cultures of leukocytes from healthy donors. The results of the DNA synthesis as determined by thymidine-^{3}H incorporation in these experiments are shown in Table IV. The leukocytes from the patients with Hodgkin's disease and those with chronic lymphocytic leukemia responded poorly to the blastogenic stimulation of PHA. These data confirm the observations of others using "blast transformation" and thymidine-^{3}H incorporation (19). The unresponsiveness of the lymphocytes could not be transmitted to normal leukocytes.

Discussion. Phytohemagglutinin stimulates the transformation to blast-like cells of lymphocytes from the thymus-dependent, peripheral lymphoid compartment. The mechanism of this stimulation is unknown; however, unresponsiveness of lymphocytes to PHA has been correlated with defective cellular immune function associated with several diseases (20). Infants with congenital rubella may have lymphocytes which fail to respond to PHA during the active phase of their disease (8–10). Rubella virus added to normal leukocytes *in vitro* inhibits PHA-induced RNA, DNA, and protein synthesis (10). In addition, the DNA synthesis of leukocyte cultures stimulated with pokeweed mitogen and the specific antigens of diphtheria–tetanus toxoids are inhibited by addition of the virus (10).

The results of the experiments reported in this study indicate that the inhibition of the blastogenic response of human lymphocytes is not a universal property of all human viruses. Inhibition of the blastogenic response was limited primarily to polio, echo, influenza A, VSV, Sendai, reo, vaccinia, and herpesviruses. Replication of viruses and serial transmission of the inhibitory effect occurred only with viruses which replicate in the cytoplasm of the cell. As exemplified with mumps virus, viral replication was not necessarily associated with the dramatic inhibition of the blastogenic response. The inhibition of DNA synthesis as measured by thymidine-^{3}H incorporation could be accounted for by cytopathic effect for such viruses as VSV, reovirus, and vaccinia virus; however, poliovirus and echovirus did not destroy the lymphocytes to a sufficient degree to account for the observed depression of thymidine-^{3}H incorporation.

Mella and Lang (21) reported that the number of cells undergoing PHA-stimulated mitosis was reduced in leukocyte cultures derived from patients with infectious hepatitis. Our findings indicate that leukocytes from hepatitis patients are hyporesponsive when stimulated with PHA. This effect was observed in leukocytes obtained early in the illness (first week of jaundice) and was detected only in cultures harvested after 3 days of *in vitro* cultivation. This observation remains unexplained and appears to represent an alteration in the metabolic response of the circulating lymphocytes since thymidine incorporation of these cells was normal after 6 days of *in vitro* cultivation. Alteration of the pattern of RNA and DNA synthesis following stimulation with PHA has also been observed in leukocytes from patients with chronic lymphatic leukemia (22). The hyporesponsiveness observed could be induced

by a factor present in the plasma, since it is known that alpha globulin, a component of normal plasma, inhibits the response of lymphocytes to PHA (18). It is also known that plasma globulins are altered during viral hepatitis. The hyporesponsiveness of the leukocytes from the hepatitis patients, however, proved to be unaltered when sera from healthy donors or from hepatitis patients were added to the cultures, indicating that the inhibitory mechanisms reside within the cells.

The inhibition of the PHA response of lymphocytes as a technique for assaying or detecting viruses appears to be of limited usefulness. A high multiplicity of infection is required to produce inhibition of the blastogenic effect for viruses which fail to replicate in leukocytes. Few of the viruses tested replicated adequately in the leukocytes to produce inhibition of the blastogenic response on serial passage. Attempts to demonstrate a transmissible inhibiting agent in specimens containing wart virus, sera from hepatitis patients, and leukocytes from patients with Hodgkin's disease and chronic lymphocytic leukemia were unsuccessful.

Summary. The PHA-stimulated leukocyte cultures from healthy donors were inoculated with representatives from picornavirus, picodnavirus, arbovirus, myxovirus, paramyxovirus, rhabdovirus, reovirus, adenovirus and papovavirus groups. Cultures were harvested after 6 days of incubation and assayed for virus multiplication and for DNA synthesis of the leukocytes by uptake of thymidine-^{3}H. Virus replication and inhibition of the PHA response of the lymphocytes by these viruses were limited primarily to viruses which replicated and assembled in the cytoplasm of the cell. Leukocytes from 15 patients with viral hepatitis were studied, and in 8 of them the cells were hyporesponsive to PHA stimulation after 3 days of incubation. This pattern was observed in all samples taken within the first week after onset of jaundice. Sera from patients with viral hepatitis when added to normal leukocyte cultures showed some inhibition of the PHA response, but the effect could not be serially passed to fresh cultures. The same pattern was obtained with human wart virus preparations. Leukocytes cultured from patients with Hodgkin's disease and with chronic lymphocytic leukemia were also hyporesponsive to PHA stimulation, but again the inhibitory effect was not transmissible to normal leukocyte cultures.

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Dietary Cholesterol and Serum Cholesterol-Esterifying Activity in Rabbits* (33629)

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Sperry (1) was the first to demonstrate that blood serum contains a cholesterol-esterifying enzyme. It is a liver produced (2) acyltransferase and the β-position of lecithin is the source of the required acyl groups (3, 4).

The activity in plasma of this enzyme, phosphatide: chloesterol fatty acid transferase, is increased in essential fatty acid (5), pantothenic acid and choline deficient rats (6) and decreased in inositol deficient rats (6). In these vitamin deficiency states, the increased activity of the enzyme is accompanied in male rats by increased concentrations of cholesterol esters in the tissues (6).

These observations suggest that the cholesterol-esterifying enzyme in plasma may serve an important role in the process of cholesterol ester deposition in the tissues. We have sought additional evidence for this concept by determining the change of activity of the enzyme in the serum of cholesterol-fed rabbits. Increased enzyme activity was observed accompanied by increased concentrations of cholesterol esters in some of the tissues.

Methods. Twelve rabbits, equally divided as to sex, with body weights in the range 2.5–3.0 kg were obtained from a commercial supplier. Two of the animals were used for the determination of the normal tissue cholesterol concentrations. Blood samples were obtained from an ear vein of each of the remaining animals at the start of the experiment and 5 days later. During this time the animals were fed a pelleted rabbit chow. [2] Sera from these blood samples were analyzed to obtain control levels of cholesterol and cholesterol-esterifying activity.

After the second blood samples were obtained, the rabbits were given the pelleted chow onto which cholesterol had been deposited from an ether solution to give a cholesterol concentration of 0.7 g/100 g of diet. The animals were continued on this diet for 6 weeks and blood samples were obtained, as above, at weekly intervals and the sera were analyzed for cholesterol and cholesterol-esterifying activity. At the end of the cholesterol feeding period, the animals were anesthetized, killed by exsanguination and samples of tissues were removed and frozen on dry ice. They were subsequently analyzed for their contents of cholesterol.

The activity of the cholesterol-esterifying enzyme in 0.50 ml of each serum sample was determined as previously described (6) using as substrate 2.0 ml of normal, heated rabbit serum containing cholesterol-1α-^{3}H.[3] Enzyme activities in the fresh experimental sera were expressed as dpm converted to cholesterol

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[2] Ralston-Purina Co., St. Louis, Missouri.

[3] Nuclear-Chicago Corp., Des Plaines, Illinois