ABNORMALITIES OF IN VITRO LYMPHOCYTE RESPONSES DURING RUBELLA VIRUS INFECTIONS

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Infants infected prenatally with rubella virus often have developmental abnormalities (1). In the wake of the rubella epidemic of 1964, many babies born of mothers having rubella infections during early pregnancy have been found to have a characteristic disease associated with prolonged excretion of the rubella virus. Clinical manifestations included the well-known triad of congenital abnormalities (1) together with such signs of active infection as hepatosplenomegaly, thrombocytopenic purpura, lymphadenopathy, and failure to thrive (2, 3). This "escalated" rubella syndrome was associated with a virus excretory phase lasting in some cases as long as 18 months, during which virus could be isolated from urine, feces, pharyngeal and conjunctival secretions, blood, spinal fluid, bone marrow, and lymph nodes (3–6). Neutralizing 19S antibodies to rubella virus together with elevated levels of IgM and decreased levels of IgG were regularly present in sera of these infants (7–9). Occasionally, such babies were found to be profoundly hypogammaglobulinemic (10, 11).

In attempting to understand the basis of continuing infection we studied the immunologic responses of such infants and found them capable of forming antibody to a variety of antigens and in several instances seemingly capable of developing and expressing delayed allergic reactions (12, 13). In the course of these investigations we found that the lymphocytes from babies with the escalated rubella syndrome did not respond normally to in vitro phytohemagglutinin (PHA) stimulation (12). Since this abnormality was associated with hepatosplenomegaly, thrombocytopenia, and failure to thrive we suggested the unresponsiveness to PHA stimulation was due to the influence of rubella virus on the lymphoid cells (12). This concept was supported by the observation that the PHA-induced DNA synthesis of normal lymphocytes was inhibited by infection with rubella virus (13).

The studies to be reported here were designed to analyze further the abnormality of peripheral leukocytes of babies during the escalated rubella syndrome. We found that leukocytes from babies which were unresponsive when first tested developed normal responsiveness to PHA stimulation at a later age when clinical manifestations of the disease had disappeared. Furthermore, normal lymphocytes infected with rubella virus showed impairment of normal DNA synthesis and PHA-induced synthesis of DNA, RNA, and structural proteins. The virus also inhibited the enhanced DNA synthesis of normal lymphocytes caused by pokeweed mitogen and tetanus-diphtheria toxoids.

Materials and Methods

Preparation of Leukocyte Cultures for DNA Analysis.—Peripheral blood leukocytes were obtained from normal adults or from babies with the congenital rubella syndrome ranging in age from 6 wk to 22 months. Heparinized blood (10 units heparin per milliliter of blood) was allowed to sediment in a plastic syringe at a 45° angle at 37°C for 1.5 hr. The sample was then placed in an upright position at room temperature for 30 min, and the cell-rich plasma supernatant transferred to a siliconized round bottom tube and centrifuged for 15–20 min at 110 *g* at room temperature. Sedimented leukocytes were resuspended and washed two times in Hanks' balanced salt solution (BSS, Grand Island Biological Co., Grand Island, N. Y.). After the final wash the cells were resuspended in 2–5 ml of Hanks' BSS, the concentration and viability determined, and the cell suspension diluted to 5×10^5 cells/ml in NCTC-109 medium containing 20% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Grand Island Biological Co.). Cell suspensions prepared by this procedure usually contained 15–20% polymorphonuclear leukocytes.

Quadruplicate cultures, each containing 2 ml of the cell suspension, were prepared for each test. The mitogens and/or other additives were added in a volume of 0.1 ml. Cultures were incubated at 37°C in a 5% CO_2 atmosphere for 1–6 days.

Preparation of Leukocyte Cultures for RNA Analysis.—Cell suspensions were prepared as described above except the final cell suspension was adjusted to 2.5×10^6 cells/ml. Duplicate cultures each containing 5×10^6 cells were prepared and incubated at 37°C in a 5% CO_2 atmosphere for 2–3 days (14).

Preparation of Leukocyte Cultures for Protein Analysis.—Cell suspensions were prepared as described above except that the final cell suspension was adjusted to 2.5×10^6 cells/ml in an isoleucine-free medium containing 100 μg/ml streptomycin and 100 units/ml penicillin (14). Each duplicate culture contained 5×10^6 cells, appropriate additives, and 2.0 μc isoleucine-^{14}C (specific activity [SA] 234 mc/mM, New England Nuclear Corp., Boston, Mass.) and was incubated at 37°C in a 5% CO_2 atmosphere for 1–2 days.

Termination of Leukocyte Cultures for DNA and RNA Analysis.—5 hr prior to termination, 2 μc of tritiated thymidine (T^3H, SA 6.7 c/mM, New England Nuclear Corp.) was added to each of three of the quadruplicate cultures of each test to be analysed for DNA synthesis. At termination 450 μg "cold" thymidine were added and the cultures were centrifuged at 700 *g* at 4°C for 15 min (15). Cell pellets were stored at −20°C until analysed. The fourth culture of each set was assayed for cell viability using the dye exclusion method (16) and for cell morphology using a Wright-Giemsa stain.

Periodically the fourth culture was pulsed with 2 μc T^3H 5 hr before termination and smears were prepared for radioautography. Radioautographs were prepared by coating the smears with Kodak NTB-2 gel emulsion, exposing them for 7–10 days, and developing them in Kodak DK-72 for 2 min (17). Smears were stained with Giemsa (18) and examined to determine the degree of labeling and to ascertain which cell types had incorporated the T^3H.

Duplicate cultures for determining RNA synthesis were terminated after 48 or 72 hr by adding either 2.8 μc tritiated uridine (U^3H, S A 6 c/mM, New England Nuclear Corp.) or 0.1 μc uridine-^{14}C (SA 20 mc/mM, New England Nuclear Corp.) per culture for 1 or 2 hr. 1 mM cold uridine was added and the cells were harvested by centrifuging at 700 *g* at 4°C for 15 min.

Cell pellets containing either labeled DNA or RNA were prepared for analysis by liquid scintillation counting according to the procedure described by Bach and Voynow (15). Cell pellets were suspended in 5 ml 5% trichloracetic acid (TCA) for 10 min, centrifuged at 700 *g* at 4°C for 20 min, dissolved in 1 ml 0.1 M NaOH, and precipitated with 4.5 ml 6.7% TCA. The digestion process of 0.1 M NaOH and 6.7% TCA was repeated and the final TCA precipitate was dissolved in 0.25 ml NCS (Nuclear Chicago Solubilizer, Nuclear Chicago Corp., Chicago, Ill.) and then dissolved in 10 ml of scintillation counting fluid (6.3 g Packard Premix P, 100 mg naphthalene, 500 ml dioxane, 500 ml absolute methanol, and 500 ml toluene). All samples were counted for 10 min on a Packard Tri-Carb scintillation counter and amount of incorporated radioactivity expressed as counts per minute (cpm) at 37% machine counting efficiency for tritium and 85% machine counting efficiency for ^{14}C.

Termination of Leukocyte Cultures for Protein Analysis.—Duplicate cultures for measuring protein synthesis were terminated after 24 or 48 hr of exposure to isoleucine-^{14}C (14). Cultures were centrifuged, supernatant fluids were decanted from the cell pellets, and both cell pellets and supernatants were dialyzed for 5 days against six changes of 0.015 M phosphate buffer pH 7.6 at 4°C. After dialysis the samples were lyophilized overnight, dissolved in 0.1 ml distilled water, 0.3 ml NCS and 10 ml scintillation counting fluid, and then counted on a Packard Tri-Carb scintillation counter as described above.

Preparation of Additives.—(*a*) Phytohemagglutinin M (PHA, Difco Laboratories, Detroit, Mich., Lot 493635) (19) was reconstituted to 5 ml with saline and used in quantities ranging from 0.1 to 0.001 volume equivalents of this PHA stock solution per culture. (*b*) Tetanus-Diphtheria Toxoid (Eli Lilly Co., Indianapolis, Ind.) was diluted to quantities of 1.5 Lf units tetanus/0.3 Lf units diphtheria per ml to 0.015 Lf units tetanus/0.003 Lf units diphtheria per ml. (*c*) Pokeweed Mitogen (PWM, Grand Island Biological Co.) (20) was reconstituted to 20 ml with saline and used in concentrations of 0.1 and 0.01 volume equivalents of stock solution per culture. (*d*) Rubella virus (Chesser strain obtained from a 4-month-old gestation fetus) was used in concentrations ranging from 2.5×10^7 to 2.5×10^3 plaque-forming units (PFU) per ml. The virus stock was prepared in monolayers of a continuous line of baby hamster kidney cells nourished in Eagle's basal medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (21). It was concentrated to approximately 2×10^7 PFU/ml by pelleting in a Spinco Model L centrifuge using a SW30/rotor at 30,000 rpm for 1.5 hr. Rubella virus was titered using a modification of the hemadsorption-negative plaque test (21). (*e*) Ultraviolet-irradiated rubella virus was obtained by irradiating a known amount of virus for 10 min at a distance of 8 inches from a 2800 A unit ultraviolet light source. (*f*) Newcastle disease virus (NDV, obtained from Dr. P. Plagemann, University of Minnesota) was assayed by a hemagglutination method (22). The virus was used at a concentration of 128 hemagglutinating units/ml.

Assay for Antibody Against Rubella Virus.—Plasma from all subjects and the final cell suspensions were checked for anti-rubella virus antibody by either the hemagglutination-inhibition test (23) or by neutralization using the hemadsorption-negative plaque test (21).

RESULTS

Responsiveness of Lymphocytes from Babies with Congenital Rubella Syndrome to PHA Stimulation in Vitro.—Table I summarizes observations made

TABLE I

Inhibition of PHA-Induced DNA Synthesis and Blastogenesis in Peripheral Leukocytes from Babies with Congenital Rubella

Subject	Initial PHA response			PHA response when clinical condition had improved	
	Age	Percent transformation or rate of DNA synthesis by cpm of T^3H-DNA	RV antibody titer*	Age	Per cent transformation or rate of DNA synthesis cpm of T^3H-DNA
J.A.	6 wk	3.0%‡	≤8	6 months	42%
T.H.	6 months	2.8 "	≤4	10 "	51"
T.H.	3 "	3.6 "			N.T.§
E.N.	2 "	6.2 "	≤4		N.T.
J.A.	1 "	9.0 "	≤4	7 "	17,000 cpm
Controls (4)	4 "	60.0 "		8 "	56%/21,000 cpm
C.B.	3 "	580 cpm‖	8	4 "	35,000
K.B.	22 "	260 "	128		N.T.
Controls (2)		15,000 "			
(Washed cells)¶					
C.B.	3 "	910 "	8		
K.B.	22 "	500	128		
(Washed cells incubated 6 days)**					
C.B.	3 "	1,000 "	8		
K.B.	22 "	465 "	128		

* Reciprocal of neutralization antibody titer by hemadsorption—negative plaque test (21).

‡ Net transformation (per cent transformation in cultures with (c̄) PHA—per cent transformation in cultures without (s̄) PHA).

§ N.T., not tested.

‖ Average of triplicate cultures. Cultures s̄ PHA stimulation averaged less than 100 cpm in all cases.

¶ Cells washed two times in Hank's BSS, resuspended in NCTC 109 with 20% fetal calf serum. Other cell preparations listed on this table were not washed.

** Cells incubated for 6 days at 37°C instead of 3 days.

when PHA responsiveness of peripheral leukocytes from seven babies with congenital rubella were examined. Each of these children had clinical signs associated with the active phase of the escalated rubella syndrome including failure to thrive, thrombocytopenia, hepatosplenomegaly, and lymphadenopathy (2, 3, 5). Six of the seven babies had neutralizing antibody titers equal to or greater than 1:4 against rubella virus, plasma from the remaining baby was

not examined. As reported earlier, babies usually under 6 months of age, affected with these clinical signs and possessing serum antibody to rubella virus possessed peripheral leukocytes which did not respond to PHA stimulation as detected by both number of transformed cells (12) and impaired rate of DNA synthesis (13). Subsequent observations on four of these babies revealed normal responsiveness to PHA stimulation after the clinical signs of disease subsided.

Furthermore, in these studies, inhibition of PHA response could not be atrributed to proteins in plasma of the babies with congenital rubella. Unwashed leukocytes and leukocytes washed two times in Hanks' BSS were obtained from two babies with congenital rubella and stimulated with PHA for 3 and 6 days. Results in Table I show that the plasma proteins removable by washing were not inhibitory to PHA stimulation of leukocytes. Nor, was an additional 3 days of culture advantageous in surmounting the inhibitory effect on PHA stimulation of these lymphocytes.

Standardization of Induced DNA Synthesis by Normal Leukocytes Stimulated with Mitogens and Specific Antigens.—To evaluate the effects of rubella virus infection upon metabolism, viability, and blastogenic transformation of human peripheral leukocytes when stimulated with mitogens or specific antigens it was necessary to determine the experimental error in these functions when cells were cultured in the absence of virus. Quadruplicate cultures of 1×10^6 leukocytes, containing less than 20% polymorphonuclear leukocytes, from three subjects on 3 consecutive days were cultured with various concentrations of PHA, PWM, or tetanus-diphtheria toxoid. Changes in cellular metabolism and blastogenesis were evaluated by assessing the amount of T^3H incorporated into DNA on the 3rd day following mitogenic stimulation. Cell viability was determined on parallel cultures on the same day.

Optimal concentration of PHA needed to stimulate 1×10^6 leukocytes as judged by both the degree of isotope incorporation and cell viability was 0.01 ml equivalents of PHA stock solution (Table II). As this concentration was increased, isotope incorporation and cell viability decreased, apparently as a consequence of cytotoxic effects of the PHA. The maximal response to PWM stimulation was one-third of the response obtained with PHA stimulation; the optimal concentration was 0.005 ml equivalents of stock PWM. Response to tetanus-diphtheria toxoid was variable but positive stimulation was obtained at concentrations of 1.5×10^{-2} Lf units of tetanus toxoid and 3×10^{-3} Lf units of diphtheria toxid as indicated by a 7.5-fold increase in DNA synthesis. Greater concentrations of these antigens were toxic to the cells.

These findings are in agreement with those of others which show that responsiveness of leukocytes to stimulations with PWM and specific antigen is less than to PHA stimulation (24–26).

Inhibition of PHA-Induced DNA Synthesis in Leukocyte Cultures with Ru-

bella Virus.—Table III summarizes observations on PHA stimulation of leukocytes from two subjects as a function of time in culture, and documents the inhibition of the PHA response obtained by the simultaneous addition of various concentrations of rubella virus to the cultures. It is evident that the maximal amount of PHA-induced DNA synthesis occurs on the 3rd day of

TABLE II

Rate of Mitogen and Specific Antigen-Induced DNA Synthesis in Peripheral Leukocytes of Normal Adult Humans

Rate of DNA synthesis, *cpm**					
Non-specific stimulations			Specific stimulations		
Mitogen dose per 1×10^6 cells	cpm T^3H-DNA after 72 hr	Cell viability	Antigen dose per 1×10^6 cells	cpm T^3H-DNA after 6 days	Cell viability
		%			%
0	140‡ ± 32§	88‖	0 Lf	430	82
0.001 ml PHA¶	155 ± 63	91	1.5×10^{-3} Lf tet./ 3×10^{-4} Lf dipht.**	1,500	84
0.01 " "	15,800 ± 6,600	88	1.5×10^{-2} Lf tet./ 3×10^{-3} Lf dipht.	3,100 ± 2,000	85
0.05 " "	10,700 ± 3,100	86	1.5×10^{-1} Lf tet./ 3×10^{-2} Lf dipht.	500 ± 350	44
0.10 " "	8,500 ± 5,800	55			
0.005 ml PWM	5,000 ± 2,400	—			
0.05 " "	2,900 ± 950	83			

* cpm, counts per minute.

‡ Average of three experiments on three subjects done in triplicate each time.

§ Standard deviation of nine values (three trials × three subjects).

‖ Average of nine cultures, determined by dye exclusion method.

¶ 0.001 ml equivalent of stock PHA solution.

** 1.5×10^{-3} Lf units tetanus toxoid/3×10^{-4} Lf units diphtheria toxoid.

culture. 1.8×10^6 plaque-forming units of rubella virus per 1×10^6 leukocytes does not impair the normal cellular function of non-PHA–stimulated cells during this period. Continuing DNA synthesis revealed by values of 59–207 cpm indicate rubella virus was neither cytotoxic nor stimulatory to washed nonsensitized cells. However, the virus caused significant inhibition of PHA-induced DNA synthesis. This effect was prominent at 1.8×10^6 PFU of virus per million cells as evidenced by 42–54% inhibition and 67–79% inhibition of DNA synthesis on days 3 and 6, respectively. Lesser virus concentration as low as two PFU of rubella virus per 100 leukocytes showed

inhibition, particularly on the 6th day of culture. This inhibition of DNA synthesis at 6 days shows that the inhibition cannot be viewed simply as a temporary decrease or delay in cellular metabolism, to be overcome with continued culture time. Rather the observations appear to reflect a persistent alteration of cells by the virus.

TABLE III

Inhibition of PHA-Induced DNA Synthesis of Peripheral Leukocytes as a Function of Concentration of Rubella Virus and Time in Culture

Concentrations of additives at 0 hr		Rate of DNA synthesis, *cpm**					
PHA	PFU of RV‡	Subject 1			Subject 2		
		Day 2	Day 3	Day 6	Day 2	Day 3	Day 6
ml							
—	—	70§	95	274	48	69	98
—	1.8×10^6	62	112	207	59	77	108
0.01	—	2315	9000	2500	2575	15,000	12,000
0.01	1.8×10^3	1550	7800	1600	2000	11,800	5,000
0.01	1.8×10^4	1250	5700	539	2000	12,900	3,100
0.01	1.8×10^5	1100	6600	673	1975	11,000	1,400
0.01	1.8×10^6	1700	5200	785	1900	6,900	2,500
		Inhibition‖					
When 1.8×10^6 PFU RV added c̄ PHA		% 44 (ns)¶	% 42 (hs)	% 67 (hs)	% 26 (s)	% 54 (s)	% 79 (hs)

* Amount of T^3H incorporated into 1×10^6 cells.

‡ Plaque-forming units of rubella virus.

§ Average of three cultures.

‖ $\%\ \text{inhibition} = \dfrac{\text{cpm for PHA} + 1.8 \times 10^6 \text{ PFU of RV}}{\text{cpm for PHA}} \times 100.$

¶ *P* value, as determined by student's t test (66). 0.1–0.05, not significant (ns); 0.05–0.02, significant (s); and 0.02–0.001, highly significant (hs).

Relationship of Inhibition of Mitogen-Induced DNA Synthesis to Time of Introduction of Virus to Leukocyte Cultures.—Rubella virus added to PHA-stimulated leukocyte cultures at 0 time or as late as 15 hr following stimulation with PHA caused significant inhibition of the mitogen-induced DNA synthesis (Table IV). When virus was introduced 24 or 48 hr after stimulation the response was not impaired significantly. However, a second susceptible period seemed to be revealed when virus was added prior to termination of the cultures, just before the T^3H pulse-labeling period.

This first observation suggests that the stimulatory effect of PHA on DNA

synthesis is reversible or at least susceptible to virus interference for at least 24 hr. This concept is complemented by other studies which found that PHA must be in the cultures for 24 hr to achieve sufficient blastogenesis (27, 28). Once the stimulated condition has been achieved, rubella virus at the concen-

TABLE IV

Inhibition of PHA-Induced DNA Synthesis by Rubella Virus Added at Various Times during Culture

Type of additives and time of additions		Rate of DNA synthesis, *cpm*			
		Experiment II*		Experiment III‡	
Mitogen	Rubella virus	Subject 1	Subject 2	Subject 3	Subject 4
0 hr	*hr*				
—	—	95§	69	117	110
—	0	112	77	57	60
PHA‖	—	6600	13,200	9300	6900
PHA	0	2500	6,600	437	528
PHA	15	3000	5,100		
PHA	24	6200	10,400		
PHA	48	5300	12,800		
PHA	64	3600	6,100	6500	6800
		Inhibition for PHA¶			
		%	%	%	%
When RV added at 0 hr		62 (s)**	50 (s)	95 (hs)	92 (hs)
When RV added at 15 hr		54 (s)	61 (s)		
When RV added at 64 hr		45 (s)	54 (s)	30 (ns)	0

* Cultures of 1×10^6 cells received 1.8×10^6 PFU of rubella virus.

‡ Cultures of 1×10^6 cells received 1.5×10^6 PFU of rubella virus.

§ Average of three cultures.

‖ 0.01 ml equivalent PHA stock solution added per culture.

¶ $\%\ \text{inhibition} = \dfrac{\text{cpm for PHA + RV at X hr}}{\text{cpm for PHA 0 hr culture}} \times 100.$

** *P* value, see Table III, ¶ footnote for key.

trations used could not interfere with aggregate DNA synthesis. However, rubella virus inhibited DNA synthesis when added at 64 hr, just before the T^3H pulse-labeling period. This observation implies that in such highly stimulated and productive cells virus infections and perhaps viral replication can temporarily alter the rate of metabolic DNA synthesis.

Table IV also reveals that different virus preparations caused differences in degree of inhibition of DNA synthesis, suggesting that the amount of impairment, although dose dependent, may also vary with the state of the virus in the preparation or upon uncontrollable variations in the amount of virus added.

Failure of Ultraviolet-Irradiated Virus to Inhibit Response of PHA-Stimulated Leukocytes.—Ultraviolet irradiation inhibits viral replication but does not alter the ability of the virus to be absorbed or to penetrate cell membranes (29). A comparison of the effect of ultraviolet-irradiated virus (UVI-virus) and live virus on PHA stimulation was made in an effort to elucidate the site of PHA activation or the site of virus inhibition of PHA-induced DNA synthesis. As noted previously, rubella virus impaired the PHA-induced stimulation. The UVI-virus failed to inhibit PHA-induced DNA synthesis in four of four cases when UVI-virus was added 3 hr before PHA stimulation and in two of four cases when UVI-virus was added simultaneously with the mitogen (Table V). In addition, the presence of UVI-virus did not prevent live virus from inhibiting the PHA response when both PHA and virus were added 3 hr after initiation of the cultures. This observation suggests that the impairment of the response to PHA is dependent upon infectious virus.

In two of eight cases studied, the irradiated virus inhibited the PHA response. All virus preparations were presumably given sufficient irradiation to inactivate the virus since Fabiyi et al. (30) have reported that 1 min of ultraviolet irradiation will destroy 4 $\log_{10}$ of virus infectivity. Our preparations were irradiated for 10 min which should have been sufficient time for inactivation. However, the possibility of multiplicity reactivation exists and could account for partial inhibition of the PHA response observed in these two instances.

Inhibition of PHA-Induced RNA Synthesis by Rubella Virus Infections.—The purpose of this study was to determine the effect of rubella virus upon PHA-induced RNA synthesis. In contrast to the lack of effect upon DNA synthesis during the first 72 hr in unstimulated cultures, the live virus caused an approximate 40–50% inhibition of RNA synthesis in six of nine non-PHA–stimulated culture sets during the first 3 days (Table VI). Cell viability in these cultures was judged to be 90–95%. PHA stimulation induced a 500–1000% increase in RNA synthesis. This response was completely inhibited by 2.5 PFU of virus per leukocyte. By contrast in another experiment, a lower concentration of virus 0.3 PFU of virus per leukocyte failed to produce significant impairment of PHA-induced RNA synthesis.

Inhibition of PHA-Induced Protein Synthesis by Rubella Virus Infection.—The rate of protein synthesis in nonstimulated and PHA-stimulated cells under the influence of rubella virus was compared by determining the amount of cell-associated (cell pellet) and cell-dissociated (supernatant) protein after 24 and 48 hr of culture. Rubella virus was found to inhibit 32–35% of the cell-associated protein synthesis in a nonstimulated cell culture (Table VII). Inhibition of cell-dissociated protein was small and not statistically significant. Following PHA stimulation, cell-associated protein synthesis was increased 85% by the 1st day and 300% by the 2nd day, in comparison with increases of 33 and 59% in cell-dissociated protein at these times. 2.5 PFU of rubella virus

per cell inhibited 50% of the PHA-induced cell-associated protein synthesis compared with 26% PHA-induced cell-dissociated protein synthesis. As in the inhibition of RNA synthesis the smaller dose of virus inhibited less PHA-induced cell-associated protein.

Leukocytes and Rubella Virus Survival in PHA-Stimulated and Nonstimu-

TABLE V

Rate of DNA Synthesis in Peripheral Leukocytes Subjected to PHA, UVI-Rubella Virus, and Rubella Virus at Various Times during Culture*

Type of additives and time of additives			Rate of DNA synthesis, *cpm*			
			Experiment II‡		Experiment III§	
PHA	UVI-RV*	Rubella virus	Subject 1	Subject 2	Subject 3	Subject 4
hr	*hr*	*hr*				
—	—	—	181	121	117	110
—	—	0	112	77	57	60
—	0	—		103	115	205
0	—	—	6500	14,700	7600	6,900
0	0	—	6500	7,600	4500	9,000
0	—	0	3400	5,600	437	528
3	—	—	7200	6,700	7100	11,000
3	0	—	6600	6,700	4900	16,000
3	—	0			429	518
3	0	3	4300	3,900	643	435
3	—	3	5900	5,500	736	692
			Inhibition			
			%	%	%	%
When UVI-RV and PHA added at 0 hr			0‖	48 (s)	51 (hs)	0
When RV and PHA added at 0 hr			48 (s)	62 (hs)	95 (hs)	92 (hs)
When UVI-RV added 3 hr before PHA			10 (ns)	0	31 (ns)	0
When RV added 3 hr before PHA					94 (hs)	95 (hs)
When UVI-RV added 3 hr before PHA and rubella virus			40 (ns)	42 (hs)	91 (hs)	96 (hs)

* Ultraviolet-irradiated rubella virus.

‡ Cultures of 1×10^6 cells received 1.8×10^6 PFU of rubella virus.

§ Cultures of 1×10^6 cells received 1.5×10^6 PFU of rubella virus.

‖ *P* value; see Table III, ¶ footnote for key.

TABLE VI

Inhibition of PHA-Induced RNA Synthesis by Peripheral Leukocytes with Rubella Virus

Type of additives and time of additions		Rate of RNA synthesis					
		Experiment I* cpm uridine-^{14}C-RNA				Experiment II‡ × 10^3 cpm uridine-^{3}H-RNA	
PHA§	Rubella virus	Subject 3		Subject 4		Subject 1	
		Day 2	Day 3	Day 2	Day 3	Day 2	Day 3
hr	*hr*	*1 hr pulse ẽ 0.1 μc uridine-^{14}C*				*1 hr pulse ẽ 2.8 μc uridine-^{3}H*	
—	—	52‖	31	58	52	30	28
—	0	38	30	33	25	45	14
0	—	331	932	—	899	252	98
0	0	34	39	42	68	209	66
		2 hr pulse ẽ 0.1 μc uridine-^{14}C				*2 hr pulse ẽ 2.8 μc uridine-^{3}H*	
	—	98		82		46	
—	0	50		49		54	
0	—	788		914		438	
0	0	79		92		306	
		Inhibition of RNA synthesis					
		%	%	%	%	%	%
In nonstimulated cells							
with RV (1 hr pulse)		27 (ns)¶	0	42 (s)	50 (s)	50	50 (s)
with RV (2 hr pulse)		49 (ns)		40 (s)		17	
In PHA-stimulated cells							
with RV (1 hr pulse)		90 (hs)	96 (hs)	—	92 (hs)	17 (ns)	33 (ns)
with RV (2 hr pulse)		90 (hs)		90 (hs)		30 (ns)	

* Cultures of 5×10^6 cells received 1.75×10^7 PFU of rubella virus. Ratio 2.5 PFU of RV/cell.

‡ Cultures of 5×10^6 cells received 1.8×10^6 PFU of rubella virus. Ratio of 0.3 PFU of RV/cell.

§ 0.01 ml equivalent PHA stock solution added per culture.

‖ Average of duplicate cultures.

¶ *P* value; see Table III, ¶ footnote for key.

lated Cultures.—This study complemented the biochemical assessment of metabolic activity in treated cells by demonstrating the effect PHA exerts upon survival of lymphocytes and rubella virus in cultures. Leukocytes from two different subjects were studied. Table VIII shows the initial drop in viable leukocytes during the first 48 hr of culture; an observation comparable with results reported by others (26, 31, 32). In the succeeding 4 days, cell survival

TABLE VII

Inhibition of PHA-Induced Cell-Associated and Cell-Dissociated Protein Synthesis by Peripheral Leukocytes Treated with Rubella Virus

Experiment and subject No.	Rate of protein synthesis*					
	Type of additives and time of additions		× 10^3 cpm cell associated ^{14}C labeled protein		× 10^3 cpm cell dissociated ^{14}C labeled protein	
	PHA	RV	Day 1	Day 2	Day 1	Day 2
	hr	*hr*				
Experiment I‡						
Subject 3	—	—	15.7§	28	5.5	9.2
	—	0	11.7	18.5	6.4	9.3
	0	—	30.5	83.8	9.4	15.7
	0	0	13.6	45	5.6	13.3
Subject 4	—	—	18	26	7.3	13.2
	—	0	0	18.7	6.3	11.7
	0	—	31.8	81.3	7.6	19.9
	0	0	18.2	37.1	6.7	12.9
Experiment II‖						
Subject 1	—	—	53.8	—	11.6	22.3
	—	0	43.7	22.3	10.9	18.9
	0	—	142	—	13.4	37.3
	0	0	103	—	11.1	28.2

Effect of PHA and RV on protein synthesis (average of subjects 3 and 4)

	Increase or decrease			
	%	%	%	%
Effect of RV on non-PHA–stimulated cell	−35 (hs)¶	−32 (hs)	−1 (ns)	−9 (ns)
PHA effect on protein synthesis	+85 (hs)	+300 (hs)	+33 (ns)	+59 (ns)
RV inhibition of PHA stimulation	−49 (hs)	−50 (hs)	−26 (ns)	−26 (ns)

* All cultures received 0.1 μc isoleucine at 0 hr.

‡ Cultures of 5×10^6 cells received 1.75×10^7 PFU of RV. Ratio 2.5 PFU of RV/cell.

§ Average of duplicate cultures.

‖ Cultures of 5×10^6 cells received 1.8×10^6 PFU of RV. Ratio 0.3 PFU of RV/cell.

¶ *P* value; see Table III, ¶ footnote for key.

was not affected by virus at concentrations up to 2.5 PFU virus per initial leukocyte. Accurate measurements of leukocyte survival were not obtained from PHA-stimulated cultures due to the PHA-induced leukoagglutination. In the second part of the Table it will be seen that 0.01 ml equivalent

stock PHA may enhance recovery of rubella virus from leukocytes for a 3-day period. By the 6th day, however, its apparent beneficial effect on virus concentration is lost. In fact, the presence of PHA may hinder virus replication, for in all cases leukocyte cultures without PHA have more virus at 6 days than at 3 days, whereas cultures with PHA show a decrease in virus content during this time period.

TABLE VIII

Survival Rate for Lymphocytes and Rubella Virus in Cultures of PHA Stimulated and Nonstimulated Peripheral Leukocyte Cultures

Days of culture	Lymphocyte and virus survival in cultures with and without PHA				
	PFU of rubella virus added per culture				
	0	2.5×10^3	2.5×10^4	2.5×10^5	2.5×10^6
	*Lymphocyte survival ± PHA**				
Day 2	106‡	96§	82	90	84
Day 3	100	108	120	115	94
Day 6	69	60	65	76	65
	Survival of rubella virus in lymphocytes + PHA				
Day 2	0	33‖	250	1250	12,250
Day 3	0	0	30	125	1,900
Day 6	0	15	150	210	2,500
	Survival of rubella virus in lymphocytes − PHA				
Day 2	0	20‖	125	1175	16,250
Day 3	0	0	165	1155	7,575
Day 6	0	25	165	500	1,750

* Each culture received 1×10^6 cells.

‡ Average of duplicate cultures from two different subjects.

§ Number of cells $\times 10^3$/ml.

‖ Plaque-forming units/ml.

Inhibition of Pokeweed Mitogen and Specific Antigen-Induced DNA Synthesis by Rubella Virus Infections.—Biochemical and morphological studies indicate that PWM and specific antigenic stimulation of leukocytes differs from that induced by PHA (24, 33). The purpose of this study was to determine whether rubella virus would inhibit responses to these stimuli as well as the responses to PHA stimulation.

Increased DNA synthesis was observed in all cultures stimulated with either PWM or diphtheria-tetanus toxoids (Table IX). The magnitude of the response was less than that following PHA stimulation and was comparable with PWM and antigen responses revealed in our standardization experiments (Table II). The addition of rubella virus impaired the stimulative effects of

PWM, but a comparison of results of impairment in PWM and PHA-stimulated cultures revealed two differences which suggest again that the responses to these two mitogens may be different. These are that PWM stimulation was

TABLE IX

Inhibition of Pokeweed Mitogen and Specific Antigen-Induced DNA Synthesis in Peripheral Leukocytes by Rubella Virus

Type of additives and time of additions			Rate of DNA synthesis, *cpm*			
Additive	Rubella virus	Cell viability	Experiment II*		Experiment III‡	
			Subject 1	Subject 2	Subject 3	Subject 4
0 hr	*hr*		*cpm on 3rd day of culture*			
	—		95§	69	117	110
—	0		112	77	87	90
PWM‖	—		1700	1800	2300	9300
PWM	0		833	650	250	170
PWM	15		1600	1600		
PWM	24		1800	1600		
PWM	48		1800	1600		
PWM	64		1600	1900		
			cpm on 6th day of culture			
—	—	89	274	193	897	648
—	0	83	207	280	35	31
Ag.¶	—	95	1050	580	1600	2900
Ag.	0	92	155	209	44	36
Effect of rubella virus on stimulated cells (Increase or decrease)						
Inhibition of PWM when RV added at 0 hr, %.....			51 (s)**	64 (hs)	99 (hs)	99 (hs)
Increase when Ag added at 0 hr, %....			170 (s)	200 (ns)	80 (ns)	350 (s)
Inhibition of Ag when RV added, at 0 hr, %.....			99 (hs)	64 (ns)	97 (hs)	99 (hs)

* Cultures of 1×10^6 cells received 1.8×10^6 PFU of rubella virus.

‡ Cultures of 1×10^6 cells received 1.5×10^6 PFU of rubella virus.

§ Average of three cultures.

‖ 0.005 ml equivalent PWM stock solution added per culture.

¶ 1.5×10^{-2} Lf tetanus/3×10^{-3} Lf diphtheria toxoid added per culture.

** *P* value; see Table III, ¶ footnote for key.

impaired only when virus was added simultaneously with PWM, and no decrease in DNA synthesis was noted when virus was added 6–7 hr before terminating the cultures.

Although less pronounced than stimulation with either mitogen, tetanus and diphtheria antigens stimulated increases of 80–350% in DNA synthesis after 6 days of culture. Rubella virus inhibited 64–99% of the specific antigen–

induced DNA synthesis. Even though virus infection did not appear to affect DNA synthesis in nonstimulated cultures during the first 3 culture days, a decrease was noted in several experiments after a longer culture period of 6 days, and this impairment of DNA synthesis was not associated with a decrease in cell viability.

DISCUSSION

Infants with congenitally acquired rubella represent an example of an intrauterine virus infection in which the host maintains an association with the virus over a prolonged period (4–6). Other examples of such infections include: congenitally acquired lymphocytic choriomeningitis (34) and virus-induced murine leukemia (35), visceral leukosis in chickens (36) and cytomegalic inclusion disease (37), and serum hepatitis in man (38). After birth, in babies with congenital rubella, virus can be isolated from multiple sites (4, 5, 7). The virus can be isolated in high frequency during the first 3 months and occasionally for as long as 16–18 months after birth (4–6, 39, 40). Many virus excretors show clinical signs of a continuing infection, and most of these infants possess high titers of neutralizing antibody to the virus (7–9). This coexistence of antibody and virus focuses attention on what we believe to be a major issue: what is the nature of the host-parasite relationship which permits persisting viral infections in the presence of neutralizing antibody?

Previous studies seemed to establish the immunologic competence of these infants and to reveal normal humoral antibody responses and cell-mediated immune responses (9, 12).[1] However, our work has shown that lymphocytes from these infants during active stages of the disease are unresponsive to PHA stimulation in vitro (12). Recently, White and Sever[2] showed that lymphocytes, from babies who had recovered from congenital rubella, had impaired in vitro responsiveness to tetanus-diphtheria toxoids. The same infants had normal humoral antibody responses to these antigens.

The observations presented in this paper provide additional data indicative that lymphoid cells of patients with the escalated rubella syndrome may be abnormal in their responses and that this abnormality is in some way a function of the activity of the disease. Patients recovering from escalated rubella syndrome whose lymphocytes have previsouly shown unresponsiveness to PHA stimulation acquire normal functions with passage of time and cessation of clinical manifestations of the disease. Our observations indicate clearly that the functional abnormality of the lymphocytes from these infants is not due to an embryonic defect of the lymphoid system or simply a secondary consequence of the disease state as might be suggested from observation of Riddle and Berenbaum (41) but is intimately related to the infection of the leukocytes by the offending virus.

However, it has been difficult to establish a positive correlation between presence of virus in vivo and impairment of the lymphocyte response to PHA stimulation. This

[1] South, M. A. Unpublished results.

[2] White, L., and J. Sever. Personal communication.

may be due in part to the difficulty in culturing virus from samples containing neutralizing antibody. It may also be due to a change in the quantity and types of lymphocytes present in the months following birth when these infants have been shown to be viremic. At birth the ratio of virus-infected cells to normal cells would be high and as age increases, the infected cell population might be diminished due to a slower growth rate (6, 42). Hence, the success in culturing a virus, and the responsiveness of a sample of lymphocytes could depend upon a possible critical ratio of virus-infected to normal cells.

The inherent problems of the in vivo system were overcome by demonstrating that rubella virus also inhibits responses of lymphocytes from normal subjects in an in vitro system. This eliminates from consideration indirect influences of disease, stress, or alterations of internal milieu and simplifies the analysis concerned with direct or indirect effects upon the response of leukocytes to mitogenic agents. At present, the knowledge of how PHA induces blastogenesis is speculative (43–47) but PHA is known to enhance metabolic activity of all cellular processes tested. These include protein synthesis (14, 43, 48, 49), mainly nonribosomal RNA synthesis (33, 50) and DNA synthesis (26, 51, 52). Our studies support these prior observations, and in addition show that the effect of rubella virus on lymphocytes is to impair DNA and RNA synthesis and structural protein synthesis in both the PHA-stimulated cells and in non-PHA–stimulated lymphocytes.

These studies seem to provide evidence that the virus exerts its effect on lymphocytes by entering the cells and not by actions on the membrane surfaces. Irradiated virus, which can be adsorbed to and penetrate cell membranes, did not, as did the live virus, regularly inhibit the mitogenic response to PHA. Even the impairment induced by live virus could be prevented if the virus and its neutralizing antibody were added simultaneously to the lymphocytes (13). However, lymphocytes have been shown to allow replication of virus within the cell and to transmit the virus to daughter cells during cell division without the virus going through a surface transmission (6, 53). Such daughter cells also appeared unresponsive to PHA stimulation. Furthermore, the live virus which inhibited PHA-induced nucleic acid synthesis, protein synthesis, and blastogenesis failed to prevent the PHA-induced agglutination of lymphocytes (54).

It was also observed that rubella virus inhibited DNA synthesis caused by pokeweed mitogen and specific antigens which probably induce their mitogenic action in cell types different from those influenced by PHA or may utilize different surface receptors to gain access to these cells. Also arguing against a surface effect is the small dose of live virus which can be effective in inhibiting the leukocyte response, for we achieved inhibitory effects with less than 1 PFU of virus per leukocyte and it is difficult to visualize how this dose of virus could be effective by a surface mechanism. Finally, the fact that both rubella virus and NDV, which have different specific membrane receptors (55), had similar inhibiting effects on PHA and pokeweed-induced responses argues

against a surface membrane action and favors the view that inhibition occurs at an intracellular location.

We interpret these observations to indicate that rubella virus enters the lymphoid cell, begins to synthesize viral RNA by taking over the ribosomal sites of the cell and thus interferes with the cell's synthesis of nucleic acids and structural proteins. This inhibition of normal metabolic functions likely interferes with the ability of the cell to undergo blast transformation in response to mitogenic stimulation and probably also interferes with their ability to exercise their normal immunologic functions.

A central issue is focused by the question: How do the abnormalities of the responses of lymphoid cells present in patients with persistent virus infections relate to the presence of virus and the integrity of the immune responses in such patients? This entire issue needs to be joined again. Although patients with escalated rubella syndrome can make antibody and seem to be able to express cellular immunity, it is important to know whether they can do so as well as can the normal child. The answer to this question may be most difficult to obtain since standards and quantitatively reliable controls are so difficult to establish, especially with respect to cell-mediated immune responses. Studies to determine the relationship of unresponsiveness of lymphoid cells to in vitro challenges with mitogens and specific antigens, to respond with humoral antibody to a battery of standard antigens, to develop or express cell-mediated immune responses, and to destroy viruses or cells containing viruses in vitro need to be carried out. Perhaps with such studies the relation of the continuing infection to immunological inadequacies, abnormalities of in vitro response of lymphocytes, and the existence of virus infections in the lymphoid system may become clear.

Inadequacies of in vitro response of lymphocytes to PHA and other stimulants are a feature of certain human diseases (52, 56–60). Our findings make it important to ascertain whether deficient responses of cells in, for example, Hodgkin's disease, chronic lymphatic leukemia, Aldrich syndrome, and even ataxia-telangiectasia could be reflections of persistent virus infections as in the escalated rubella syndrome.

Perhaps, some diseases associated with impaired in vitro responsiveness of lymphocytes may be indicative of virus-cell relationships similar to those under study here. The pathogenesis of certain neoplastic diseases, such as virus-induced murine leukemias and perhaps adenovirus-induced tumors in hamsters may involve temporary viral induced unresponsiveness in lymphocytes, thus preventing the host from mounting an effective immune response against the malignant cells (61).

In vitro studies have shown impaired responses with lymphocytes from patients with infectious hepatitis (62) and infectious mononucleosis (32, 63). It is conceivable that such virus infections may, like the rubella and NDV, have the capacity to produce comparable inadequacies by depressing the cells own mechanism for protein and nucleic acid synthesis. If this be the case it might be possible to employ this influence on lymphoid cells as a means of detecting viruses and viral influences not detectable by other means.

Many new experimental approaches are suggested by these findings. For example, it seems obvious that a broad spectrum of in vitro immune responses of lymphocytes can be altered by infection with certain viruses. Would it be possible to infect lymphoid cells and thus interfere with their ability to exercise such in vivo responses as those involved in creating graft versus host reactions or homologous disease? Interfering with this type of a reaction with either a nonvirulent or nonpathogenic virus might make it possible, at least temporarily, to abrogate unwanted lymphocyte reactions but yet allow the lymphoid cells to eventually recover and be able to exercise defensive functions. Such influences could be most salutory in patients with lymphopenic agammaglobulinemia syndromes (64, 65).

Whatever the fundamental nature of the virus lymphoid cell relationship responsible for the interference with mitogenic and specific antigen-induced responses of lymphocytes may be, it seems clear that a new and potentially important virus host relationship has been brought into focus by these studies and this relationship deserves further inquiry.

SUMMARY

In vitro rubella virus infections of lymphocytes from normal adult humans impaired their responsiveness to phytohemagglutinin (PHA) stimulations; a situation which seemed analogous to the PHA unresponsiveness of peripheral lymphocytes from babies with the congenital rubella syndrome. Such in vitro viral infection of normal cells also decreased the synthesis of normal nucleic acids and structural proteins, and abrogated the enhanced DNA synthesis induced by pokeweed and specific antigen stimulations. Furthermore, it was shown that live rubella virus, but not ultraviolet-irradiated virus, was necessary for the impaired mitogenic responses of normal leukocytes.

These observations are interpreted to favor the view that the virus achieves its inhibitory effect on the action of mitogens by interference either directly or indirectly at an intracellular site. Such an action could reduce the functional potential of lymphocytes and impair their effectiveness as immunologically competent cells or as effectors in immunologic reactions.

Addendum. Since completion of these experiments and submission of this manuscript, attempts were made to passage the effects of virus-induced PHA unresponsiveness by adding the supernatant of virus-infected cultures to new cell cultures. During this work it was found that one of the rubella virus stock preparations used in determining the effects of virus on PHA-induced macromolecular synthesis was contaminated with PPLO. As Copperman and Morton (67) have shown that PPLO impairs PHA-lymphocyte responsiveness, the additive effect of inhibition by PPLO must be considered as a complicating factor in interpretating the virus impairment of PHA-induced macromolecular synthesis. However, rubella virus preparations shown to be free of PPLO by standard laboratory procedures have been found to in-

hibit the PHA-induced DNA synthesis as described in the text. In addition, certain other RNA viruses such as mumps and polio viruses which were not contaminated with PPLO produced a similar inhibition of lymphocyte responsiveness.

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