

# CELL-MEDIATED IMMUNITY IN RUBELLA ASSAYED BY CYTOTOXICITY OF SUPERNATANTS FROM RUBELLA VIRUS-STIMULATED HUMAN LYMPHOCYTE CULTURES

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## SUMMARY

Rubella virus-stimulated lymphocytes from rubella-seropositive donors produced in the culture medium cytotoxic activity with preferential action against rubella-infected over uninfected target cells. The ability of lymphocytes to produce the cytotoxic activity upon stimulation by rubella virus correlated with the humoral rubella-immunity status, i.e. no such cytotoxic activity developed in the supernatants of lymphocyte cultures of rubella-seronegative donors. Stimulation of lymphocytes from seropositive donors by rubella virus was also detected by thymidine incorporation, but the correlation of lymphocyte responsiveness to the humoral rubella antibody status was not so clear as in the cytotoxicity assay. Conversion of lymphocytes from unresponsive to responsive to rubella virus following natural rubella infection and after rubella vaccination was demonstrated using both methods. Following vaccination rubella-specific cell-mediated immunity first became demonstrable at 14 days. The responsiveness of lymphocytes to phytohaemagglutinin (PHA) after rubella vaccination was followed by studying thymidine uptake and the ability of lymphocytes to produce lymphotoxin. By both tests marked suppression of PHA response occurred at days 3 and 7 after vaccination.

## INTRODUCTION

Lymphocytes from immune humans and animals can be activated by viral antigens *in vitro*, but relatively little is known about the development of cell-mediated immunity (CMI) as measured by lymphocyte stimulation during viral infections. Rosenberg, Farber & Notkins (1972) demonstrated in rabbits that sensitization of lymphocytes to *in vitro* stimulation by the virus occurred within 3 days after immunization with inactivated herpes simplex virus. The kinetics of development of cytotoxic killer cells in relation to humoral immunity have been characterized in tumor-virus systems (e.g. Skurzak *et al.*, 1972;

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Lamon *et al.*, 1973). As for human viral infections, Steele *et al.* (1973a, b) reported appearance of cytotoxic peripheral blood lymphocytes against rubella-infected target cells 3 days after immunization of humans with live attenuated rubella vaccine.

Delayed hypersensitivity is impaired during some viral infections, such as measles (Pirquet, 1908), and also following vaccination with live attenuated measles (Brody, Overfield & Hammes, 1964) and rubella virus vaccines (Berkovich, Steiner & Steiner, 1969). There is also preliminary information about suppression of responsiveness of lymphocytes *in vitro* to phytohaemagglutinin (PHA) after rubella vaccination (Midulla, Businco & Moschini, 1972; McMorro *et al.*, 1974). Therefore it is important to consider occurrence of virus-induced immunosuppression in *in vitro* studies of CMI in these viral infections.

In the preceding paper (Kanra & Vesikari, 1974) we demonstrated that lymphocytes from rubella-immune subjects activated *in vitro* by rubella virus developed in the culture medium cytotoxic activity, which preferentially killed rubella-infected target cells. Though the nature of the cytotoxic activity remains to be characterized, it was believed that the ability of lymphocytes to produce the activity could be used as a test for CMI in rubella. In the present work we have compared the lymphocyte stimulation-cytotoxicity test to lymphocyte stimulation measured by thymidine uptake as an indicator of CMI to rubella virus.

In addition we report preliminary data about the development of CMI after rubella infection and rubella vaccination, as measured by the same parameters. The occurrence of rubella virus-induced immunosuppression was followed simultaneously by studying the responsiveness of lymphocytes to PHA, assayed by thymidine incorporation. As another indicator, the ability of lymphocytes to produce cytotoxic activity against uninfected target cells was followed after PHA stimulation.

## MATERIALS AND METHODS

### *Patients*

For testing of rubella immunity blood was obtained from ten healthy adults, nine women and one man, aged 23–38 years. Three of the subjects had no recollection of rubella in the past; one had a questionable history of rubella in childhood; and six had positive histories. In two cases the past rubella infection had also been diagnosed serologically. Rubella antibody status was determined by the haemagglutination-inhibition antibody (HI) test according to the standard procedure of the Center for Disease Control (1970).

Development of immunity could be followed in two young women during the course of natural rubella. Rubella vaccinees in this study included two young women with initial rubella HI-antibody titre less than 1:8 and one with an equivocal ( $\leq 1:8$ ) antibody titre. Each vaccinee received RA 27/3 strain of live attenuated rubella vaccine (Burroughs Wellcome, Canada) subcutaneously.

### *Lymphocyte cultures*

Cultures from peripheral leucocytes were established according to the macromethod of Valentine (1971). In brief,  $1.5 \times 10^6$  small lymphocytes were cultured in 3 ml of minimum essential medium (Grand Island Biological Company, Buffalo, N.Y.) supplemented with 15% autologous plasma. Antigen-stimulated cultures were incubated for 144 hr and PHA-stimulated cultures for 72 hr in an atmosphere containing 5% CO<sub>2</sub> before harvest of the supernatant for use in the cytotoxicity test.

For the thymidine incorporation test 0.1  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]thymidine (New England Nuclear) (specific activity 50  $\mu\text{Ci}/\text{mmole}$ ) was added to triplicate (or duplicate, in some cases when the yield of lymphocytes was not sufficient) cultures 18 hr prior to termination of the tests. The trichloroacetic acid precipitable radioactivity was measured by scintillation counting in a Packard Tri-Carb counter, and the mean counts of the triplicate or duplicate cultures were recorded. The results are expressed as stimulation ratios compared to cultures with no addition of antigen.

Rubella virus antigen preparation, control antigen and PHA were described in the preceding paper (Kanra & Vesikari, 1974). It is to be emphasized that u.v. light-inactivated partially purified rubella virus was used in all lymphocyte stimulation studies.

#### *Cytotoxicity test*

The method, a modification of that of Takasugi & Klein (1970), was also described in detail in the preceding paper (Kanra & Vesikari, 1974).

## RESULTS

Table 1 summarizes the results of serological screening and studies for CMI to rubella in the ten donors tested. Six donors were unequivocally seropositive. Each had a history of clinical rubella; in two cases the infection was serologically confirmed as rubella at the time of illness. One donor was questionably seropositive; her rubella HI titre was  $\leq 1:8$  on repeated tests. Three donors were rubella-seronegative on repeated testing.

Lymphocytes from all rubella-seropositive donors showed an increased rate of thymidine uptake at day 6 upon stimulation by rubella virus, and the stimulation ratios over unstimulated or cultures with no added antigen ranged from 3.4-fold to 10-fold under the standard conditions (Table 1). The equivocally seropositive subject also showed 4.4-fold stimulation by thymidine uptake, and one of the seronegative donors showed 2.7-fold stimulation on day 6. Lymphocytes from the other two seronegative donors were not stimulated by rubella virus.

Cultures with BHK21 control antigen preparation showed in most cases slightly increased thymidine uptake compared to control cultures. The greatest observed stimulation ratio over the control cultures was 2.1-fold in one case, and in two other cases 1.5-fold stimulation was detected (Table 1). All the donors responded to stimulation by PHA and the stimulation ratios over 'unstimulated' cultures ranged from 11-fold to 86-fold in the 72 hr cultures.

Cytotoxicity tests on rubella-infected and uninfected target cells were performed using the supernatant fluids at day 6 from lymphocyte cultures kept in parallel with those used for the thymidine incorporation tests. Cytotoxic activity against rubella-infected target cells was produced in the rubella virus-stimulated lymphocyte cultures of all rubella-seropositive donors, including the one with equivocal rubella HI antibody titre (Table 1). In the latter case cytotoxic activity against rubella-infected target cells was 28%, in the other rubella-immune subjects the cytotoxic activity ranged from 46% to 67%. Cytotoxic activity of supernatants from rubella-stimulated lymphocyte cultures of the seropositive subjects against uninfected target cells did not exceed 7% (Table 1).

Supernatant fluids from lymphocyte cultures or rubella-seronegative donors cultured in the presence or absence of rubella virus showed little or no cytotoxic activity against

TABLE 1. Screening for cell-mediated immunity to rubella by thymidine incorporation test and cytotoxicity test using supernatants from rubella virus-stimulated lymphocyte cultures against rubella-infected and uninfected target cells. The results of thymidine uptake test are expressed as stimulation ratios compared to unstimulated cultures. The results of cytotoxicity test are given as percentage cytotoxicity (see Materials and Methods section)

Donor	History of rubella	Rubella HI titre	[ <sup>14</sup> C]thymidine uptake stimulation ratio			Cytotoxicity test (% cytotoxicity)						
			Rubella virus		BHK control	Rubella-infected target cells			Uninfected target cells			
			Rubella virus	BHK control		Rubella virus	BHK control	No addition	Rubella virus	BHK control	No addition	
			%	%	%	%	%	%	%	%	%	%
G.M.	7 years	512	10.0	1.1	1.1	52	0	0	0	1	3	
R.S.	10 years	256	10.0	2.1	2.1	67	0	2	6	6	3	
N.C.	18 years	256	3.4	1.4	1.4	51	1	3	5	3	2	
S.F.	15 years	128	5.8	1.5	1.5	65	1	1	7	+4	0	
E.R.	19 years	64	8.8	1.5	1.5	46	3	3	2	3	3	
T.V.	5 years	64	3.6	1.2	1.2	47	1	0	0	+3	1	
G.K.	As child?	±8	4.4	n.d.	n.d.	28	4	+3	+3	2	0	
L.B.	None	<8	2.7	n.d.	n.d.	1	8	+1	+2	7	+1	
R.D.	None	<8	0.5	n.d.	n.d.	+1	n.d.	4	4	n.d.	7	
A.Y.	None	<8	0.6	0.7	0.7	0	n.d.	0	1	n.d.	0	

n.d. = Not determined.

rubella-infected or uninfected target cells. Lymphocyte culture supernatants from rubella seropositive or seronegative donors kept with BHK21 control antigen preparation did not show any more cytotoxic activity than unstimulated cultures against either kind of target cells (Table 1).

Development of CMI to rubella was followed in two cases of natural rubella infection. Only a few specimens could be obtained, and no BHK21 cell control antigen preparation was available at this stage of the study.

Both patients showed good PHA response but no response to rubella antigen on the second day after onset of rash, as measured by thymidine uptake and, in one patient, cytotoxicity tests. A decreased PHA response was seen in one patient on day 4 and in the other on day 12. The former one also showed response to rubella antigen on day 4, but this was detected only by thymidine uptake and not by cytotoxicity (Table 2).

By day 45 both patients had seroconverted. The response of lymphocytes to rubella virus was low, 2.8-fold and 1.3-fold, respectively, as measured by thymidine incorporation at 144 hr. Rubella-stimulated lymphocyte cultures of both patients now produced cytotoxic activity against rubella-infected target cells of 62% and 46% respectively, but no cytotoxic activity against uninfected target cells (Table 2). Cytotoxic activity against infected and uninfected target cells was demonstrable in the supernatant fluid from PHA-stimulated cultures of both patients. Therefore the PHA responsiveness of lymphocytes appeared to have returned to normal, though in one case this was not studied by thymidine uptake.

The development of CMI to rubella and changes in the PHA responsiveness of lymphocytes were followed after rubella vaccination. Two rubella-seronegative women (L.B. and R.D. in Table 1) and an equivocally seropositive one (G.K.) were given RA27/3 strain of live attenuated rubella virus vaccine subcutaneously, and lymphocyte cultures were established on days 3, 7, 14 and 28. No changes in the rubella antibody titre occurred by day 14; by day 28 the two seronegative vaccinees had seroconverted and both had rubella HI antibody titres of 1:32. No clear booster effect was seen in the third vaccinee: at day 28 the rubella HI antibody titre was 1:8.

The results of thymidine incorporation tests and cytotoxicity tests of each vaccinee are summarized in Table 3, and some selected data are graphically illustrated in Fig. 1. Studies with BHK21 cell control antigen were done in single cultures only, and therefore the results of thymidine incorporation tests are not presented in Table 3. Instead the results of cytotoxicity assays of these culture supernatants are shown. These supernatants never showed cytotoxicity above 8% against rubella-infected or uninfected target cells.

Development of CMI to rubella as measured by the present techniques followed a somewhat different course in each of the vaccinees (Table 1 and Fig. 1). In case L.B. no rubella-induced cytotoxic activity was detected by day 7, some specific cytotoxicity (15% killing of rubella-infected target cells) was demonstrable at day 14, and by day 28 the lymphocytes responded by production of cytotoxic activity at a level similar to that seen previously in rubella-immune subjects. Conversion of lymphocytes to rubella responsiveness was also seen by thymidine uptake assay, though this patient's lymphocytes had also had some responsiveness to rubella antigen prior to the immunization (Table 3). In the second vaccinee (R.D.), who also had seroconverted, only slight specific stimulation of lymphocytes by rubella virus was seen by day 28 both in the cytotoxicity and thymidine uptake tests. The vaccinee was further tested at day 39, but her lymphocytes were still unresponsive to rubella virus. Lymphocytes from the third vaccinee (G.K.), initially equivo-

TABLE 2. Development of cell-mediated immunity to rubella and changes in the PHA-responsiveness of lymphocytes during natural rubella infection. Lymphocyte stimulation by rubella virus and PHA was measured by thymidine uptake and the results are expressed as stimulation ratios compared to unstimulated cultures. Cytotoxicity test was performed using supernatants of rubella-stimulated, PHA-stimulated, and unstimulated lymphocyte cultures against rubella-infected and uninfected target cells

Patient	Time after onset of rash	Rubella HI titre	[ <sup>14</sup> C]thymidine uptake stimulation ratio		Cytotoxicity test (% cytotoxicity)					
			Rubella virus	PHA	Rubella-infected target cells			Uninfected target cells		
			Rubella virus %	PHA %	Rubella virus %	No addition %	PHA %	Rubella virus %	No addition %	PHA %
P.R.	2 days	<8	1.2	108	+1	2	48	2	3	42
	4 days	16	4.5	5.3	0	0	0	8	3	5
	45 days	128	2.8	n.d.	62	5	74*	3	2	73*
L.C.	2 days	<8	1.0	17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	12 days	256	0.7	0.2	7	7	2	6	5	3
	45 days	128	1.3	22	46	0	74	3	+2	75

n.d. = Not determined.

\* Six day culture. In other PHA-stimulated lymphocyte cultures the cytotoxicity was assayed at 72 hr.

TABLE 3. Lymphocyte response by thymidine uptake and by cytotoxicity test to rubella virus and to PHA in three recipients of RA 27/3 strain of live attenuated rubella vaccine

Donor	Time after vaccination	Rubella HI titre	[ <sup>14</sup> C]thymidine uptake stimulation ratio		Cytotoxicity test (% cytotoxicity)									
			Rubella virus	PHA	Rubella-infected target cells					Uninfected target cells				
			Rubella virus %	PHA %	Rubella virus %	BHK control %	No addition %	PHA %	Rubella virus %	BHK control %	No addition %	PHA %		
L.B.	Pre-vacc.	<8	2.7	31	1	8	1	83	2	7	+1	60		
	3 days	<8	2.5	2.6	7	3	0	13	3	3	3	13		
	7 days	<8	1.2	4.2	+2	0	+1	18	1	0	+2	24		
	14 days	<8	1.5	14	15	4	2	74	0	2	1	65		
28 days	32	32	5.2	6.3	88	1	0	82	+2	0	0	76		
R.D.	Pre-vacc.	<8	0.5	15	+1	4	4	78	4	7	7	70		
	3 days	<8	1	0.9	0	2	1	5	1	4	2	9		
	7 days	<8	1.6	5.2	1	1	+2	59	2	1	1	39		
	14 days	<8	1.3	10	1	3	2	70	3	3	2	65		
	28 days	32	32	1.8	10	6	1	67	4	+2	2	60		
39 days	n.t.	n.t.	1.5	10										
G.K.	Pre-vacc.	±8	4.4	86	28	4	+3	63	+3	2	0	64		
	3 days	±8	0.6	1.2	6	+1	+1	5	3	+1	0	12		
	7 days	±8	1.6	1.6	10	3	1	7	2	3	1	10		
	14 days	8	0.8	4	11	4	3	69	3	2	3	68		
28 days	8	2.4	6	89	0	1	80	+3	+1	+1	74			

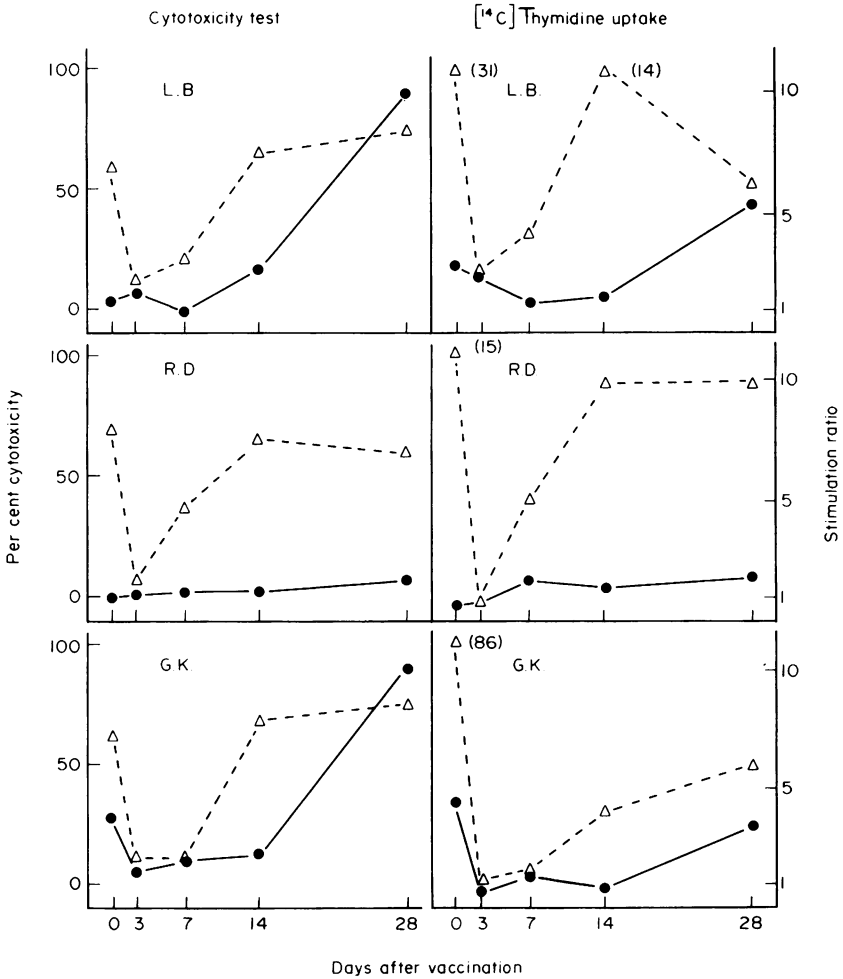


FIG. 1. Response of lymphocytes to stimulation by rubella virus (●) and by PHA (Δ) in three vaccinees given RA27/3 rubella vaccine subcutaneously. Parallel figures represent the test results for the same vaccinee by cytotoxicity test and by incorporation of [<sup>14</sup>C]thymidine. The cytotoxic effect of lymphocyte culture supernatants of rubella-stimulated cultures on rubella-infected target cells is shown by solid lines, and that of PHA-stimulated culture supernatants on uninfected target cells by dashed lines. The results of thymidine uptake tests are shown as stimulation ratios compared to unstimulated lymphocyte cultures.

cally seropositive, but responsive to rubella virus before the vaccination, were unresponsive to rubella virus by thymidine uptake test and almost unresponsive by the cytotoxicity assay on days 3, 7 and 14. At day 28 her lymphocytes responded again to rubella virus in the thymidine uptake test. More cytotoxic activity against rubella-infected target cells was detected now than before vaccination. Lymphocyte culture supernatants from cultures with rubella virus, control BHK21 antigen or with no addition of any of the vaccinees showed no significant cytotoxic activity against uninfected target cells (Table 3).

The changes in the PHA responsiveness of lymphocytes were quite uniform in all three



vaccinees (Table 3 and Fig. 1). By thymidine incorporation test a peak suppression of PHA-response was detected at day 3 with subsequent return of responsiveness by day 14 or 28. PHA-stimulated lymphocytes did not produce significant measurable cytotoxic activity on day 3. In one case there was detectable cytotoxic activity on day 7, and all three vaccinees showed prevaccination level of cytotoxic activity production upon PHA stimulation on day 14 (Table 3 and Fig. 1). In conclusion, all vaccinees showed rather uniform, short-lasting immunosuppression following rubella vaccination as measured by the PHA-responsiveness of lymphocytes.

## DISCUSSION

Though the nature of the cytotoxic activity developing in the supernatants of rubella-stimulated lymphocyte cultures remains to be characterized, it appeared from the preceding study that an immune-specific release of one or more lymphokines was involved. Therefore it seemed possible to try using the formation of cytotoxic activity by rubella-stimulated lymphocytes as an *in vitro* correlate of CMI in rubella, and to compare it to lymphocyte activation measured by thymidine uptake.

At present the optimal conditions for lymphocyte stimulation by rubella virus as measured by the rate of DNA synthesis and for the production of cytotoxic activity have not been thoroughly worked out. Consequently it is possible to do comparisons between the tests only with regard to the particular test conditions used in this study. In two previous works from other laboratories on lymphocyte stimulation by rubella antigens considerably different methods and especially different antigens have been applied, which in turn makes direct comparisons with these difficult (Simons & Fitzgerald, 1968; Smith, Chess & Mardiney, 1973).

Appearance of cytotoxic activity in the supernatants of lymphocyte cultures kept in the presence of inactivated rubella virus appeared to distinguish the rubella immunes from rubella susceptibles. In the small number of subjects tested so far there have been no false positives, and none of the rubella-seropositive individuals tested have failed to respond by production of cytotoxic activity upon stimulation of lymphocytes by rubella virus.

Activation of lymphocytes by rubella virus as measured with thymidine uptake could also be seen in all rubella-seropositive donors. The stimulation ratios were variable and generally low. Among the three seronegative donors one showed a low level of stimulation of lymphocytes. We suspect that this response may reflect the crudeness of the antigen preparation and does not represent the specific response to rubella virus antigen. Moreover, a corresponding 'purified' control antigen also caused some stimulation of lymphocytes. Therefore it appeared that under the present test conditions the lymphocyte stimulation test was useful but not quite distinctive for the detection of rubella immunity. This corresponds well with the results of Smith *et al.* (1973), who, using a crude commercial rubella antigen preparation, failed to detect complete correlation between the humoral immunity status and responsiveness of lymphocytes to rubella antigen. It has been our experience (Valentine & Vesikari, unpublished observations) that crude cellular antigens prepared in the same fashion as standard virus antigens cause even more stimulation of lymphocytes than the control antigen of this study, which was processed like the partially purified virus. It is to be emphasized that such control antigen-stimulated lymphocyte cultures did not appear to produce cytotoxic activity against rubella-infected target cells.

In immunization studies with animals various manifestations of developing CMI to the viral antigen have been detected early, within a few days, after inoculation. The assay methods have included lymphocyte stimulation measured by thymidine uptake (Rosenberg *et al.*, 1972), cytotoxic activity of spleen cells against (mumps) virus-infected target cells (Speel, Osborn & Walker, 1968), and production by lymphocytes upon *in vitro* incubation virus antigen of soluble cytotoxic factor (Oldstone & Dixon, 1970), or macrophage migration-inhibition factor (Feinstone, Beachey & Rytel, 1969). Steele *et al.* (1973a, b) reported development of CMI to rubella as early as 3 days after immunization of children with rubella vaccine. The CMI was assayed by incubation of patient's lymphocytes  $^{51}\text{Cr}$  with labelled rubella-infected BHK21 cells followed by measurement of released isotope.

In the present work we did not study direct cell-associated cytotoxicity in the rubella patients or rubella vaccinees, which might have been an earlier indicator of CMI than the tests applied. It was previously shown that lymphoblast proliferation *in vivo* occurred after rubella vaccination with a peak as late as at 2 weeks, but association of the blast proliferation with specific lymphocyte functions, such as cytotoxicity, was not studied (Lalla, Vesikari & Virolainen, 1973). It appears that the time of blast proliferation corresponded with the return of PHA responsiveness in the present study.

Specific response of lymphocytes to rubella virus seemed to develop in two vaccinees at the same time as the suppression of PHA response was over. In a third vaccinee, however, the lymphocytes remained unresponsive to rubella virus over a period of at least 39 days although she had seroconverted and her PHA response had returned to normal. Therefore one should be cautious in correlating the appearance of specific CMI to rubella to rubella-induced suppression of PHA response. Lymphocytes may not be activated by rubella virus *in vitro* during the initial suppression of PHA response or, possibly, T-cell function, but in addition there may be other factors determining the development of CMI to rubella after infection.

Though temporary suppression of PHA-response after rubella vaccination has been reported previously (Midulla *et al.*, 1972) the present few cases illustrate more clearly than before its timing and transient nature. The mechanism by which rubella virus suppresses lymphocyte responsiveness is not known, but it has been shown that in *in vitro* conditions rubella virus appears to be more suppressive than many other viruses (Willems, Melnick & Rawls, 1969). The assay of PHA response by cytotoxic activity production was a 'side-product' of studies on rubella-activated lymphocytes, but it might contribute as an additional assay method to other work on PHA response. In a recent study (McMorrow *et al.*, 1974) a dissociation between two assays of PHA response, the thymidine uptake and mitotic rate of stimulated lymphocytes, was detected in rubella vaccinees when changes in the lymphocyte responsiveness were followed.

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