Lymphocyte Responses to Rubella Antigen and Phytohemagglutinin after Administration of the RA 27/3 Strain of Live Attenuated Rubella Vaccine

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Received for publication 16 December 1974

Lymphocyte phytohemagglutinin (PHA) responsiveness was found suppressed in both rubella sero-negative and sero-positive recipients of RA 27/3 strain of live attenuated rubella vaccine; the suppression was readily demonstrable only when a suboptimal dose of PHA was applied in the test. Lymphocytes from sero-negative vaccinees, which initially showed little or no in vitro response to concentrated rubella virus, became responsive after vaccination by day 21, when the highest sensitization to rubella antigen was seen. In the sero-positive vaccinees, lymphocytes responded to rubella antigen in vitro before vaccination, and in most cases vaccination did not result in significant changes in lymphocyte response. These results suggest that rubella vaccination leads to temporarily increased lymphocyte reactivity to rubella antigen, and the increased lymphocyte response to specific antigen may occur at the time of mild suppression of PHA response.

Rubella virus has an inhibitory effect on lymphocyte responsiveness to mitogens in vitro (7, 15; A. Morag, B. Morag, J. M. Bernstein, K. Buetner, and P. Ogra, submitted for publication). Attenuated strains of rubella virus appear to be less suppressive in vitro than wild-type virus (14). Evidence suggestive of a correlation between the level of attenuation and the degree of immunosuppression also in vivo was found previously in a study with experimental rubella vaccines (10). In the present study we have studied whether suppression of lymphocyte phytohemagglutinin (PHA) response after rubella vaccination is detectable by the thymidine incorporation method, and whether this observation can be extended to challenge rubella sero-positive individuals with rubella vaccine.

The existence and development of specific cell-mediated immunity (CMI) to rubella virus has been studied by several methods. Simons and Fitzgerald first showed that human lymphocytes can be stimulated in vitro by inactivated rubella antigen (15). In subsequent studies involving different antigen preparations, good but not complete correlation between humoral rubella antibody status and in vitro lymphocyte response to rubella antigen was found (12, 16, 21). The appearance of CMI to rubella after vaccination has been demonstrated by studying lymphocyte-mediated cytotoxicity against rubella-infected cells (18) and, more recently, by rubella-specific release of macrophage migration inhibition factor (4) and by lymphocyte in vitro thymidine uptake (12).

In the present study, changes of in vitro lymphocyte responsiveness to rubella antigen were followed in rubella sero-negative as well as sero-positive vaccinees, and development of rubella-specific CMI was correlated with studies of lymphocyte PHA response.

MATERIALS AND METHODS

Subjects and vaccines. Two groups of vaccinees were studied. The first one consisted of four student nurses who were rubella sero-negative (titer less than 1:8) by hemagglutination inhibition antibody test (2). This group received subcutaneously RA 27/3 strain rubella vaccine (Wellcome Research Laboratories, Beckenham, Kent, England), lot Ru 4/17. When titrated in African green monkey kidney cells by the method of challenge with Echovirus type 11 (13), the lot had a titer 10^4.0 mean tissue culture infective doses per ml. The second group consisted of four rubella sero-positive young female hospital employees. They received the same strain of rubella vaccine, but lot RS 2/2, which titered 10^4.2 mean tissue culture infective doses per ml in African green monkey kidney cells. No clinical reactions were observed in either group of vaccinees.

Preparation of leukocyte cultures. Heparinized blood was allowed to settle 90 to 120 min at 37 C, and leukocyte-rich plasma was separated. The cells were washed twice in minimal essential medium, and
resuspended in minimal essential medium containing 15% autologous plasma to give a concentration of 500,000 small mononuclear cells per ml. The cultures were established by dividing the cell suspension in 1-ml aliquots in capped plastic tubes (12 by 75 mm) (Falcon Plastics), and incubated at 37°C in an atmosphere containing 5% CO₂.

In the first group of vaccinees lymphocyte cultures were studied at 1, 3, 7, 11, 21, and 43 days after vaccination, and the plasma used to supplement the culture media was collected at the same time. In addition, parallel leukocyte cultures for PHA stimulation were established using autologous plasma taken before vaccination and stored at −20°C. In the second group lymphocyte cultures were established on days 1, 5, 12, 19, 26, and 54. Additional leukocyte cultures were prepared, whenever a sufficient amount of cells were available, after removal of adherent cells by 90 min of incubation of leukocyte suspension in plastic tissue culture flasks.

**Thymidine incorporation assay.** ³H-labeled thymidine (New England Nuclear Corp.; specific activity >50 mCi/mmol) was diluted in minimal essential medium to a concentration of 0.2 μCi/ml. Prior to termination of leukocyte cultures, 0.2 ml of this dilution was added to each culture. The length of pulse was 16 h for PHA cultures and 4 h for cultures with rubella or control antigen. PHA cultures were harvested at 40 h and antigen cultures at 96 h.

In the first part of the study the leukocyte cultures were harvested using trichloracetic acid precipitation followed by washing in methanol and dissolving the precipitate in hydroxide of hyamine before scintillation counting (20). In the second part the harvesting was done using a multiple cell culture harvester (Skatron A/S, Lierbyen, Norway), which disrupts the cells with distilled water and collects and rinses the insoluble material on a glass fiber filter. In comparative studies we did not observe significant differences in scintillation counting results between the two harvesting methods. The scintillation counting in both cases was done with Econofluor (New England Nuclear Corp.) in a Packard Tri-Carb counter.

**PHA.** PHA P, lot 3110-56 (Difco Laboratories), was used throughout the study. The dilutions used on most occasions, 1:200 and 1:1,000, were prepared prior to the vaccination of each group, divided into small aliquots, and stored at −70°C until used. Diluted PHA was added in the amount of 0.1 ml per leukocyte culture.

**Rubella virus and antigen preparations.** Rubella virus was propagated in BHK-21 cell roller bottle cultures and concentrated by centrifugation against a 20%/65% sucrose cushion (19). After dialysis against tris(hydroxymethyl)aminomethane-buffered saline the antigen preparations titered 1:256 or 1:512 by hemagglutination test. Rubella virus antigens were inactivated by exposure to ultraviolet light for 60 min.

A control BHK-21 cell antigen preparation was made from supernatant fluid of uninfected cells by a procedure similar to that used in virus concentration. For the first group of vaccinees a 10% cell homogenate of uninfected human embryonic lung cells (NYU 32 line) was used as the control antigen.

All antigens were applied in a volume of 0.1 ml per leukocyte culture.

**Statistical analysis.** The tabulated stimulation ratios were tested by two-way analysis of variance, determining whether there were significant variations between the days after vaccination or between the individuals in the study.

**RESULTS**

**Lymphocyte response to PHA.** Figure 1 illustrates a dose response curve to PHA under the present test conditions. Of these concentrations, a dilution of 1:200 was chosen to present optimal concentration and a dilution of 1:1,000 to present suboptimal dose of PHA in the vaccination studies.

In the sero-negative rubella vaccinees, no significant decrease in lymphocyte response to optimal PHA concentration was seen, though some low stimulation ratios were recorded between days 1 and 11 (Table 1). By using the suboptimal PHA concentration, decrease in response was seen in all vaccinees. The follow-up remained incomplete, however, since only two vaccinees were tested beyond 43 days (Table 1). It appeared that this mild suppression could be quite long lasting, since by day 43 all vaccinees still showed low response to suboptimal dose of PHA.

To study the possible effect of humoral factors on PHA responsiveness after vaccination, parallel cultures were established using autologous plasma collected prior to the vaccination and plasma taken at the time of preparing the lymphocyte culture. This procedure was followed up to 21 days after vaccination, and at no time was there any significant difference in the lymphocyte response to PHA between cultures supplemented with either kind of plasma.

In the sero-positive vaccinees little or no effect by rubella vaccination on PHA response was seen when the optimal dose was used in the test. In contrast, all vaccinees showed suppression of response to suboptimal dose of PHA (Table 2). The suppression was most marked on days 5 and 12 and was seen in all subjects regardless of the prevaccination level of lymphocyte response to this dilution of PHA. By day 26 the PHA responses had returned to normal in all sero-positive vaccinees.

In this part of the study parallel leukocyte cultures for PHA stimulation were established before and after the removal of adherent cells on plastic surface, whenever a sufficient yield of lymphocytes was obtained to allow this. When depleted from adherent cells, the total counts in PHA-stimulated as well as unstimulated leukocyte cultures were lower, but the stimulation
ratios remained similar to those in regular leukocyte cultures, suggesting that the effect of rubella vaccination was not mediated through adherent cells (macrophages).

**Lymphocyte response to rubella virus antigen.** The kinetics of lymphocyte response and optimal dose of rubella antigen were first studied in several rubella sero-positive donors. In general, a low dilution of antigen gave the best response, and no stimulation of lymphocytes was seen when an antigen preparation was diluted to 1:128 or further. In the present study a dilution of 1:2 was applied throughout (1:2 dilution of concentrated rubella virus preparation in a volume of 0.1 ml added to 1 ml of lymphocyte culture). In time course experiments with this dose of antigen, a typical one of which is shown in Fig. 2, the highest stimulation ratio was usually seen on day 4. Still higher uptake of thymidine was often detected in rubella antigen-stimulated cultures on days 5 and 6, but this increase was accompanied by increased background activity in unstimulated cultures. Therefore, in this study, all antigen cultures were harvested at 96 h.

In the sero-negative vaccinees, the prevaccination lymphocyte stimulation ratios ranged from 0.7- to 1.9-fold with rubella antigen and from 0.7- to 1.4-fold with control antigen (Fig. 3). After vaccination the lymphocyte responses remained at the same level through day 11. A significant change was seen on day 21 when all the vaccinees showed a high, 4.5- to 12-fold, response to rubella antigen. Rubella hemagglutination-inhibition antibodies also appeared in all vaccinees between days 11 and 21. None of the vaccinees had any increased response to control fibroblast cell antigen. The peak responsiveness was followed by a decline as early as day 43 (Fig. 3). When the stimulation ratio data were studied using a two-way analysis of variance, the increased response to rubella antigen on day 21 was significant at a level P < 0.01.

Of the sero-positive vaccinees, two had had natural rubella and two had been vaccinated 1 and 2 years earlier with live, attenuated rubella virus vaccine. Lymphocytes from all vaccinees showed response to stimulation with rubella antigen, and the stimulation ratios ranged from 2.9- to 7.4-fold (Fig. 4). One subject also showed a 2.7-fold response to control BHK-21 cell antigen; the others had no response. After challenge with RA 27/3 rubella vaccine virus, the lymphocyte responses to rubella antigen

![Fig. 1. Dose response of lymphocytes to PHA in one of the test subjects before vaccination. The cultures were established in 15% autologous plasma with 500,000 lymphocytes per ml of culture and PHA was added in a volume of 0.1 ml. [3H]thymidine was added at 24 h, and the cultures were harvested at 40 h.](image)

<table>
<thead>
<tr>
<th>Table 1. Lymphocyte PHA responses after rubella vaccination in four sero-negative vaccineesa</th>
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a The vaccinees received RA 27/3 strain of rubella vaccine subcutaneously. In vitro PHA response was measured and assayed by incorporation of [3H]thymidine at 40 h. Results are expressed as stimulation ratios counted from the means of triplicate cultures.

a ND, Not done.
TABLE 2. Lymphocyte PHA response after rubella vaccination in four sero-positive vaccinees

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*See Table 1 for details.

* ND, Not done.

Fig. 2. Lymphocyte response of a rubella sero-positive subject to ultraviolet-inactivated rubella virus antigen at different times of culture. The values represent stimulation ratios between cultures stimulated with optimal dose of rubella antigen and unstimulated cultures, as calculated from the mean counts of triplicate cultures. [14C]thymidine was added to the cultures 4 h before harvesting.

showed some decrease or remained unchanged. (Fig. 4). The vaccinee who initially responded to control antigen showed increased sensitization of lymphocytes, both to rubella antigen and control antigen on day 12, with return to prevaccination level by day 26 (Fig. 5). No vaccinee developed a significant booster antibody response.

Transiently increased in vitro sensitization of lymphocytes to rubella antigen after vaccination was demonstrated in all sero-negative and in one out of four sero-positive subjects. A mild suppression of PHA response occurred both in sero-negative and sero-positive vaccinees. The increased lymphocyte response to specific antigen occurred at the same time as the decreased response to mitogen (Fig. 5).

DISCUSSION

Our present findings confirm that live attenuated rubella virus vaccine has a mild, transient suppressive effect on CMI, and show that this effect is demonstrable even in initially rubella sero-positive subjects. The fact that suppression of in vitro lymphocyte response to PHA was unequivocally detected only using a suboptimal dose of PHA may explain why in other studies either no suppression was found (6, 8) or the effect was seen only in some individuals (10, 11). Only mild suppression of PHA response in vivo by rubella vaccination is expectable in view of the studies of Savel and Phillips (14), who showed that rubella vaccine viruses are less inhibitory than wild-type virus to PHA stimulation of lymphocytes in vitro.

While PHA response of lymphocytes as measured by incorporation of thymidine is not
The mechanism of suppression of lymphocyte mitogen response by rubella virus remains to be established. We were unable to show that suppression was mediated by adherent cells, and therefore it appeared that rubella virus directly affected the lymphocytes. This differs from results described previously for poliovirus: Soontiëns and van der Veen (17) showed that poliovirus blocked the enhancing effect of macrophages on the PHA response of lymphocytes. Since in rubella PHA response was also affected in the immune individuals, it seemed that either a very small amount of virus was required for this or that the suppressive effect could be mediated by virus-antibody complexes. In rabbits, Lee and Sigel showed that rubella antigen/immunoglobulin M antibody complexes formed in vitro inhibited the PHA response of lymphocytes (9). Our present study failed to detect any humoral cause for PHA suppression in the vaccinees. It would, however, have been more appropriate to continue the study beyond 21 days.

Specific lymphocyte in vitro response to rubella antigen developed in the sero-negative vaccinees between days 11 and 21, apparently at a time there was a mild suppression of PHA response (to suboptimal dose). Since rubella antibodies had also appeared by day 21, it was not possible to demonstrate the exact time sequence of these phenomena. Honeyman et al. (4) recently showed that migration inhibition factor production from rubella-sensitized lymphocytes preceded humoral antibody formation after rubella vaccination. In analogy, since the lymphocyte in vitro stimulation is likely to represent a measure of T-cell function, it is conceivable that using different timing one might see the lymphocyte in vitro response to antedate the antibody response.

Also, in accordance with the results of Honeyman et al. (4), the in vitro reactivity of lymphocytes to rubella virus appeared to decrease soon after vaccination, in this case by 43 days. The latter stimulation ratio values were of the same order as we have seen in individuals with a history of rubella for years in the past, for example the sero-positive vaccinees in this study. It seems that in most cases it is possible to distinguish between rubella-immune and susceptible subjects by the lymphocyte in vitro stimulation test. However, occasionally only low degree of stimulation is seen in sero-positive subjects and conversely some stimulation by rubella antigen may be detected in sero-negative subjects (12, 16, 21). Therefore, it appears too early to assess the significance of in vitro lymphocyte stimulation test in this respect.
Similarly, it remains to be established whether increased lymphocyte sensitization after vaccination could occur to anything but rubella virus antigens. We observed increased responsiveness of lymphocytes to control antigen in only one sero-positive vaccinee, but it is possible that use of more controls could have revealed more reactivity against tissue antigens. Field et al. (3) recently demonstrated that sensitization of lymphocytes to encephalitogenic factor occurred in natural measles, and the course of sensitization followed that seen for measles antigen.

Finally one might want to speculate with the biological significance of cellular immune response in rubella. The questions that immediately arise include its role in the various clinical complications of natural infection as well as vaccination, particularly in relation to prolonged persistence of the virus in the organism. Also, in view of the recent report of Honeymon and Menser (5), it would be interesting to evaluate a possible correlation between CMI response in rubella and occurrence of HL-A tissue type antigens.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant NO1 AI 92222 from the National Institute of Allergy and Infectious Diseases.

We thank Louis Z. Cooper, Director of the Pediatric Service at The Roosevelt Hospital, for continuing support and interest in these studies. The technical assistance of Laura Selub and Thomas Byrne is gratefully acknowledged.

LITERATURE CITED