Transformation of Lymphocytes From Immunized Rh(D)-Negative Subjects by Rh(D) Isoantigen

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Since immune memory in Rh(D)-negative isoimmunized subjects remains through life, even in the absence of measurable anti-Rh(D), we investigated the transformation of lymphocytes from such donors by Rh(D) antigen. The time lapse from the last stimulus was up to 13 years. Mononuclear cells from immunized women were stimulated by Rh(D)-positive erythrocyte stroma. Maximum transformation was observed on the sixth day of culture with a stroma protein concentration of 8 μg/mL of culture medium. The stimulation index (SI) in cells from 11 immunized women was 6.8 ± 3.1 (mean ± SD), with a range from 3.1 to 15.0. In five different sets of control cultures, the SI ranged from 0.9 ± 0.2 to 1.3 ± 0.4. There was no overlap between stimulated and control cultures. No anti-D could be demonstrated in the serum of four of the 11 immunized cases studied. Also, transformation was observed in mononuclear cells from Rh(D)-negative immunized women with Rh(D)-positive erythrocytes. The findings demonstrate that lymphocytes from isoimmunized Rh(D)-negative subjects maintain the immune memory and are transformed in vitro by the Rh(D) isoantigen.

THE MOST IMPORTANT blood incompatibility in pregnancy is that involving the Rh factor. In approximately 9% of all pregnancies, an Rh(D)-negative white woman will carry an Rh(D)-positive fetus. In previously immunized women, small amounts of fetal blood that enter the maternal circulation are sufficient to elicit an anamnestic response. Although not seen routinely, the initial maternal response is the formation of IgM anti-D, followed by the production of IgG anti-D. The titer of IgG anti-D and less frequently of IgM anti-D have been used for the identification of immunized women during pregnancy or for a period of time following sensitization. However, in addition to the difficulties in detecting the Rh antibodies and the naturally occurring drop in antibody titers with time, it appears that immunization may occur in some cases although the agglutination tests remain negative.3

Rosette-forming cells with Rh(D)-positive erythrocytes have been identified in the peripheral blood of Rh(D)-negative isoimmunized women2,3 and immunized men.4 Application of the rosette test demonstrated that serologic tests cannot identify all sensitized women.5 Also, the mononuclear cells of only a small number of isoimmunized women formed rosettes.2

Since Rh(D)-negative isoimmunized individuals maintain the immune memory practically throughout life, we investigated in vitro the response of lymphocytes derived from immunized women to Rh(D) antigen.

MATERIALS AND METHODS

Eleven Rh(D)-negative women immunized to Rh(D)-positive isoantigen, by previous pregnancies, were used in the study. All women had at least one delivery or in utero death of an Rh(D)-positive child affected with hemolytic disease of the newborn and positive indirect anti-human globulin tests during and just after delivery. Eleven Rh(D)-negative, nontransfused, nonimmunized men and 11 Rh(D)-positive subjects, nonimmunized to any blood group antigens, were used as controls. Six of the Rh(D)-positive subjects were men and five were women. None of them were transfused. The ABO blood group of the control subjects was matched with that of the corresponding isoimmunized women.

Twenty milliliters of heparinized blood (10 μM heparin, fenol-free, per milliliter of blood) was obtained by venopuncture. Mononuclear cells (mainly lymphocytes) were separated by the Ficoll-hypaque system.2 Cells were washed three times with RPMI 1640 medium and subsequently cultivated at 1 × 10⁶ cells/mL in the same medium supplemented with 10% fetal calf serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. When indicated, stroma from Rh(D)-positive or from Rh(D)-negative, group O, erythrocytes was added to the medium from the beginning of cell culturing. Stroma was prepared by successive treatment in hypotonic phosphate buffers from 0.1 to 0.01 mol/L, pH 7.4, and centrifugations at 1,470 to 20,000 g according to the method of Pinteric et al.5 The same preparation of cell stroma was used in all experiments. In some experiments, lymphocyte-free whole erythrocytes instead of erythrocyte stroma were used, which were isolated by centrifugation in Ficoll-hypaque. The ratio of lymphocytes to erythrocytes in the culture tube (Greiner Laborteknik, West Germany) was 1:1.

Thymidine incorporation into DNA was measured by adding 10 μCi [³H]-thymidine (Amersham; 5 Ci/mmol) to 1 mL of cell suspension after five days of culturing. Cultures were stopped 18 hours later by transferring the tubes in ice water and washing the cell with cold (4°C) saline. Cell pellets were treated with cold 10% trichloroacetic acid (TCA) for 30 minutes and the TCA-insoluble fractions were collected on glass fiber filters, 0.45 μm (Millipore Co). The filter discs were washed three times with 10 mL 10% TCA and the amount of radioactivity incorporated was determined in a liquid scintillation counter.

All cultures were carried out in triplicate. Each set of sample tubes was cultured in parallel with five sets of control tubes (see below). The stimulation index (SI) was calculated by dividing the cpm in the cultures with antigen by the cpm in cultures of the same cells without antigen.7 Rh antibodies in the serum were detected by the antiglobulin technique.4 Protein was determined by the method of Lowry et al.4 Student’s t test was applied for the statistical analysis of the data.

RESULTS

The optimal dose of erythrocyte stroma for anti-Rh(D) response was defined by adding to the culture medium

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Fig 1. Dose curve of lymphocyte stimulation ([3H]-thymidine incorporation) by erythrocyte stroma. — mononuclear cells from an immunized Rh(D)-negative woman with Rh(D)-positive erythrocyte stroma; — mononuclear cells from the same woman with Rh(D)-negative erythrocyte stroma; — mononuclear cells from an nonimmunized Rh(D)-negative woman with Rh(D)-positive erythrocyte stroma.

Fig 2. Time curve of lymphocyte stimulation ([3H]-thymidine incorporation) by erythrocyte stroma (8 µg/mL). Symbols as in Fig 1.

Fig 3. Response of lymphocytes assessed by [3H]-thymidine uptake on day 6 after an 18-hour [3H]-thymidine pulse, expressed as SI. The difference between the SI of the cells from the Rh(D)-negative immunized women with Rh(D)-positive erythrocyte stroma and the SI from the five control groups is highly significant (P < .001). Rh(−) I, mononuclear cells from immunized Rh(D)-negative women; Rh(−) NI, mononuclear cells from nonimmunized Rh(D)-negative subjects; Rh(D), mononuclear cells from Rh(D)-positive subjects; Rh(D) ES, erythrocyte stroma from a Rh(D)-positive subject; Rh(−) ES, erythrocyte stroma from a Rh(D)-negative subject.
### DISCUSSION

The mitogenic effect of Rh(D)-positive erythrocytes and erythrocyte stroma on the lymphocytes of Rh(D)-negative women previously immunized by Rh(D)-positive fetuses demonstrates that the lymphocyte maintains the immune memory and has the ability to become transformed in vitro when incubated with the antigen. Lymphocyte stimulation occurred even in cases with nondetectable anti-Rh(D) in the blood. The specificity of the reaction was shown by the failure to stimulate the same lymphocytes with stroma or intact erythrocytes from Rh(D)-negative subjects, as well as the absence of a response in the five sets of control cultures used in the study. As expected, the stimulation of lymphocytes from immunized donors by the specific antigen, ie, Rh(D)-positive erythrocytes and stroma, was a weak one when compared with that caused by the strong nonspecific mitogen PHA.

The sensitivity of the method is suggested by the fact that in the immunized women studied, the SI was statistically significantly greater than in the control cultures, with no overlapping between the test cultures and the control groups. Although in this study the longest period of time lapsed from the last known in vivo stimulus and the in vitro transformation was 13 years, it seems reasonable to assume that the ability of the lymphocytes for immune response will last longer, at least throughout the reproductive era. However, since the lowest SIs were observed in three cases immunized 7 to 13 years earlier, further investigation of this issue is warranted.

It is known that some Rh(D)-negative subjects appear to be incapable of making any response whatsoever to the Rh(D) antigen, when tested for serum anti-D. At present, it is not known whether or not the lymphocytes of such "nonresponders" will be transformed when cultivated with Rh(D) antigen. Significant perturbations of the normal pattern of lymphocyte responses to the mitogens phytohemagglutinin, concanavalin-A, and pokeweed were observed in both "responders" and "nonresponders" after booster immunizations. This may indicate that the lymphocytes of both types recognize the rhesus antigen and will be transformed in vitro by this antigen.

Mononuclear cells forming rosettes in vitro with Rh(D)-positive erythrocytes have been identified in the blood of pregnant Rh(D)-negative isoimmunized women containing saline anti-D agglutinins (short-lived IgM antibodies) suggesting that these women were recently stimulated by fetal erythrocytes. On the contrary, no rosette-forming cells were found in pregnant women with high indirect Coombs' titers of anti-D (IgG long-lived antibodies) or Rh(D)-negative nonpregnant women previously immunized to the Rh(D) isoantigen. It has been found in animal studies that this immunocytoadherence technique detects antibody-forming B cells, which can fix the corresponding erythrocyte at their surface.

Although no experiments were performed to identify the population of lymphocytes that underwent proliferative response, it is most likely that they were memory maintaining T cells that became transformed by the Rh(D) isoantigen. This explains the finding that using the technique reported here, women with or without circulating anti-D that were immunized many years in the past were identified, whereas the rosetting technique would not detect these patients. Experiments using T-cell enriched and B-cell enriched populations will solve this issue.

Application of the rosette immunocytoadherence test on Rh(D)-negative women six to 14 days after an abortion, demonstrated rosette-forming cells in 8.5%, whereas a rise in Rh(D) antibody titer was found only in 3% of them. Possibly, in vitro transformation of the lymphocytes of such cases with Rh(D) isoantigen will identify an even larger number of immunized women than that found by using other techniques. It is not clear why Katz and Marcus failed to demonstrate transformation of the lymphocytes derived from immunized women by the Rh(D) isoantigen using both whole erythrocytes and erythrocyte stroma. However, it should be noted that for the preparation of the erythrocyte stroma, trypsin was used, which could affect the antigenicity of the preparation. Also, there is no mention of lymphocyte removal from the packaged erythrocytes used for lymphocyte stimulation. In this case, lymphocyte cross-stimulation would cover any transformation of the lymphocytes from the Rh(D)-negative isoimmunized women by the Rh(D)-positive erythrocytes.

It is accepted that following a first pregnancy, some Rh(D)-negative women will be primarily immunized, but will not produce sufficient anti-D to be detectable serologically. In these patients, a secondary response from another pregnancy will result in the appearance of anti-D. Also, the Rh(D) antibodies decline in the serum of immunized women to nonmeasurable levels after a variable period of time. In such cases, in which anti-D can no longer be demonstrated serologically, a transfusion given many years after the last stimulus may evoke a hemolytic transfusion reaction.
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Considering the in vivo response, it can be assumed that in cases of immunized subjects with no detectable anti-D, cultivation of the lymphocytes with Rh(D) antigen will result in lymphocyte transformation.

Although the use of Rh immune globuline has been very effective in reducing the number of cases affected with hemolytic disease of the newborn, new women are sensitized and, therefore, some cases still occur. This technique provides an additional tool for the diagnosis of these women and may be helpful in the management of such cases.

REFERENCES

4. Elson CJ, Bradley J: The immunocyto-adherence of Rh(D) positive erythrocytes to mononucleated cells from the peripheral blood of Rhesus isoimmunized individuals. Int Arch Allergy 40:382, 1971
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