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

By Anastasia Varvarigou-Frima, Stephanos Mantagos, and Nicholas G. Beratis

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.

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stimulation was evident after 24 hours of culturing (Fig 2). Observed on the sixth day of culture, even though lymphocyte activation was found when lymphocytes from previously immunized subjects were cultured in the presence of stroma. Maximum lymphocyte response was obtained at a stroma protein per milliliter of medium. Lymphocyte stimulation was obtained with sensitized Rh(D)-negative, cells from Rh(D)-negative nonimmunized subjects with Rh(D)-positive stroma, cells from Rh(D)-negative nonimmunized subjects with Rh(D)-positive stroma, cells from Rh(D)-negative subjects with Rh(D)-positive stroma, and cells from Rh(D)-positive subjects with Rh(D)-negative stroma, the SI ranged from 0.9 ± 0.2 to 1.3 ± 0.4 (Fig 3). The difference between the SI of the lymphocytes from previously immunized Rh(D)-negative subjects cultured in the presence of Rh(D)-positive stroma and each of the control groups was highly significant (P < .001, \( t = 4.7 \) to 5.3), with no overlapping between the test cultures and the control groups. The mean SI in mononuclear cell cultures containing phytohemagglutinin (PHA) “M” (Difco) at a concentration of 0.4 mg/mL of medium was 50 after 72 hours of culturing.

Similarly, transformation was observed in lymphocytes obtained from three Rh(D)-negative isoimmunized women, when cultivated in the presence of Rh(D)-positive, lymphocyte-free, whole erythrocytes. The mean SI was 4.7 (range, 3.6 to 6.0), whereas the mean SI of all samples in the five control groups mentioned above was 1.4 (range, 0.8 to 2.0).

No significant linear correlation was observed between the magnitude of the lymphocyte proliferating response and the time lapse from last maternal immunization (r = 0.684).

LYMPHOCYTE TRANSFORMATION BY RH(D) ANTIGEN

![Graph](image1)

**Fig 1.** Dose curve of lymphocyte stimulation ([\(^{3}H\)]-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.

Various amounts of stroma, ranging from 0.2 to 400 \( \mu \)g of stroma protein per milliliter of medium. Lymphocyte stimulation was observed only with sensitized Rh(D)-negative lymphocytes cultured in the presence of Rh(D)-positive stroma. Maximum lymphocyte response was obtained at a protein stroma concentration of 8 \( \mu \)g/mL of culture medium. Higher concentrations caused an inhibitory effect. No lymphocyte activation was found when lymphocytes from previously immunized subjects were cultured in the presence of Rh(D)-negative stroma or lymphocytes from Rh(D)-negative nonimmunized subjects were cultured with Rh(D)-positive stroma (Fig 1). Peak responses of lymphocytes were observed on the sixth day of culture, even though lymphocyte stimulation was evident after 24 hours of culturing (Fig 2).

Lymphocyte stimulation by Rh(D) antigen was obtained from mononuclear cells derived from Rh(D)-negative isoimmunized women, when cultured in the presence of Rh(D)-positive stroma. The SI in 11 cases studied was 6.8 ± 3.1 (mean ± SD), with a range from 3.1 to 15.0. In the five types of control cultures, cells from Rh(D)-negative immunized subjects with Rh(D)-negative stroma, cells from Rh(D)-negative nonimmunized subjects with Rh(D)-positive stroma, cells from Rh(D)-negative nonimmunized subjects with Rh(D)-negative stroma, cells from Rh(D)-positive subjects with Rh(D)-positive stroma, and cells from Rh(D)-positive subjects with Rh(D)-negative stroma, the SI ranged from 0.9 ± 0.2 to 1.3 ± 0.4 (Fig 3). The difference between the SI of the lymphocytes from previously immunized Rh(D)-negative subjects cultured in the presence of Rh(D)-positive stroma and each of the control groups was highly significant (P < .001, \( t = 4.7 \) to 5.3), with no overlapping between the test cultures and the control groups. The mean SI in mononuclear cell cultures containing phytohemagglutinin (PHA) “M” (Difco) at a concentration of 0.4 mg/mL of medium was 50 after 72 hours of culturing.

Similarly, transformation was observed in lymphocytes obtained from three Rh(D)-negative isoimmunized women, when cultivated in the presence of Rh(D)-positive, lymphocyte-free, whole erythrocytes. The mean SI was 4.7 (range, 3.6 to 6.0), whereas the mean SI of all samples in the five control groups mentioned above was 1.4 (range, 0.8 to 2.0).

No significant linear correlation was observed between the magnitude of the lymphocyte proliferating response and the time lapse from last maternal immunization (r = 0.684).

**Fig 2.** Time curve of lymphocyte stimulation ([\(^{3}H\)]-thymidine incorporation) by erythrocyte stroma (8 \( \mu \)g/mL). Symbols as in Fig 1.

**Fig 3.** Response of lymphocytes assessed by [\(^{3}H\)]-thymidine uptake on day 6 after an 18-hour [\(^{3}H\)]-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(D) ES, erythrocyte stroma from a Rh(D)-negative subject.
Table 1. Time From Last Known Stimulus, ABO Blood Group, Presence or Absence of Anti-D, and SI of Lymphocytes from Rh(D)-Negative Isoimmunized Women by Rh(D) Antigen

<table>
<thead>
<tr>
<th>No.</th>
<th>Time</th>
<th>ABO</th>
<th>Anti-D</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pregnant*</td>
<td>O</td>
<td>+</td>
<td>15.0</td>
</tr>
<tr>
<td>2</td>
<td>3 mo</td>
<td>O</td>
<td>-</td>
<td>6.5</td>
</tr>
<tr>
<td>3</td>
<td>8 mo</td>
<td>O</td>
<td>+</td>
<td>7.3</td>
</tr>
<tr>
<td>4</td>
<td>8 mo</td>
<td>O</td>
<td>+</td>
<td>6.0</td>
</tr>
<tr>
<td>5</td>
<td>1 yr</td>
<td>O</td>
<td>+</td>
<td>6.5</td>
</tr>
<tr>
<td>6</td>
<td>2 yr</td>
<td>A</td>
<td>-</td>
<td>7.2</td>
</tr>
<tr>
<td>7</td>
<td>3 yr</td>
<td>A</td>
<td>+</td>
<td>8.0</td>
</tr>
<tr>
<td>8</td>
<td>6 yr</td>
<td>A</td>
<td>-</td>
<td>8.2</td>
</tr>
<tr>
<td>9</td>
<td>7 yr</td>
<td>B</td>
<td>+</td>
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</tr>
<tr>
<td>10</td>
<td>8 yr</td>
<td>O</td>
<td>-</td>
<td>4.0</td>
</tr>
<tr>
<td>11</td>
<td>13 yr</td>
<td>O</td>
<td>+</td>
<td>3.1</td>
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</table>

Abbreviations: SI, stimulation index; +, presence of anti-D; -, non-detectable anti-D.
*At study 22 weeks pregnant; first immunized 8 years earlier.

However, the lowest SIs (3.1 to 4.0) were found in cases no. 9, 10, and 11 in which the last known stimulus had occurred 7 to 13 years earlier. Also, there was no relationship between the magnitude of the SI and the presence of antibody, as indicated by the absence of anti-D in the serum of four of the 11 isoimmunized women studied (Table 1).

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 rhesus 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

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Transformation of lymphocytes from immunized Rh(D)-negative subjects by Rh(D) isoantigen

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