Rosette Formation and Inhibition in Cervical Dysplasia and Carcinoma in Situ

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SUMMARY

Total and early rosettes and rosette inhibition were measured in patients with cervical dysplasia and carcinoma in situ. Both total and early rosettes were significantly depressed in patients with carcinoma in situ; early rosettes were also significantly lower than were controls in women with severe dysplasia. Rosette inhibition titers were increased in most patients with moderate dysplasia and in all patients with either severe dysplasia or carcinoma in situ. Thus, the rosette inhibition test may be useful in detecting, in a precancerous state, patients at risk for cancer.

INTRODUCTION

Spontaneous rosette formation with SRBC by peripheral blood lymphocytes has been used as a measure of thymus-derived lymphocytes (15). However, the ratio of SRBC to lymphocytes and the time of incubation markedly affect the percentage of RFC detected (5, 13). Thus, Wybran and Fudenberg (16) have used an SRBC:lymphocyte ratio of 8:1 and a short incubation for detection of lymphocytes considered to have high-affinity receptors for SRBC. These cells termed "active" or "early" RFC have been shown to correlate better than "total" RFC with the clinical status of patients (9, 16, 17) and may represent lymphocytes committed more immediately to cell-mediated immune responses (6).

When RFC are preincubated with ALS, their ability to form rosettes is inhibited (4, 14). Although it is not known whether this characteristic represents a marker for a specific thymus-dependent lymphocyte subpopulation, clinical studies suggest that rosette inhibition may have direct relevance to the evaluation of the integrity of the cell-mediated immune response. The rosette inhibition test was first applied to the clinical management of renal transplant patients (2, 11) and, more recently, was used to detect abnormalities in certain neoplastic diseases, including Burkitt's lymphoma (8) and carcinoma of the lung (7).

Since the rosette inhibition test appeared to discriminate between benign and malignant pulmonary lesions (7), it was of interest to determine its applicability in precancerous conditions. For this purpose women with cervical dysplasia and CIS were studied. In view of the association between genital herpes simplex virus infections and cervical neoplasia (12), patients with frequently recurring genital herpes were also included.

MATERIALS AND METHODS

The study population included: (a) women in whom a recent biopsy or cone demonstrated cervical dysplasia (mild, moderate, or severe) or CIS, before initiation of treatment; (b) healthy women with no evidence of cytological abnormalities in recent Papanicolaou cervical smears (the controls were matched with the patients as closely as possible in age, race, and socioeconomic status); (c) individuals with recurrent genital herpes of at least 2 years duration with at least 4 recurrent episodes/year. Peripheral blood was collected by venipuncture into heparinized containers, and the lymphocytes were separated by centrifugation through a Ficoll-Hypaque gradient (3). The interface cells were recovered, washed twice with HBSS, and resuspended in HBSS supplemented with FCS. For identification of mononuclear phagocytes, 0.1 ml of 1% latex (Dow Chemical Co., Indianapolis, Ind.; 10%, 0.801 μm in diameter) was added to the lymphocyte suspension and incubated at 37° for 30 min. The mixture was then layered over 1 ml of FCS and centrifuged at 200 × g for 5 min to remove free latex particles. The lymphocytes were washed once in HBSS, and the solution was adjusted to a final concentration of 1 × 10^6/ml.

Fresh SRBC in Alsever's solution were obtained each week. Before use they were washed 3 times in HBSS, counted, and diluted to the appropriate concentration. Early RFC were measured by the method of Wybran and Fudenberg (17). After preincubation for 60 min at 37°, 0.1 ml of the lymphocyte suspension was mixed with SRBC to give an SRBC:lymphocyte ratio of 8:1. After centrifugation at 200 × g for 5 min, the cells were gently resuspended, and the proportion of RFC in a sample of 200 cells was determined. A rosette was defined as a lymphocyte with 3 or more adherent SRBC.
The rabbit anti-human lymphocyte serum used in the rosette inhibition test was obtained from C. B. Reimer and E. Ades, Center for Disease Control, Atlanta, Ga. Serial double dilutions of ALS in HBSS were prepared, and to 0.1 ml of each dilution was added an equal volume of the lymphocyte suspension. HBSS was used as a control. After incubation at room temperature for 30 min, 0.2 ml of FCS and 0.1 ml of SRBC, equivalent to an SRBC:lymphocyte ratio of 40:1, were added; the cells were pelleted by centrifugation at 200 x g for 5 min and incubated at room temperature for a further 2 hr. Rosettes were counted as described above. All determinations were performed in duplicate. The percentage inhibition compared to controls (total RFC) was determined for each dilution, and the 25% inhibition point was determined by graphical methods. The results were expressed as the reciprocal of the titer giving 25% inhibition.

Bone marrow-derived lymphocytes were identified by use of the technique by which SRBC are coated with antibody and complement. Five ml of a 5% suspension of SRBC were incubated at 37° for 30 min with an equal volume of a 1:500 dilution of rabbit IgM antibody to SRBC and washed 3 times in HBSS. The pellet was resuspended in 5 ml of a 1:1000 dilution of fresh guinea pig serum, incubated for a further 30 min at 37° and washed 3 times in HBSS. The rosettes of SRBC coated with antibody and complement were formed by mixing 0.1 ml of the lymphocyte suspension (1 x 10⁶ cells) with 0.1 ml of a 1% suspension of the prepared SRBC, followed by incubation for 10 min at 37°. The cells were pelleted by centrifugation at 1000 rpm for 5 min and resuspended gently; the RFC were counted.

The statistical significance of comparisons between various patient groups was evaluated by 1-way analysis of various techniques. Mean values were compared with Scheffe's test. Because rosette inhibition titers did not conform to a normal distribution, they were converted to logarithms for statistical analysis.

RESULTS

Rosette formation and inhibition in the various patient groups are shown in Table 1. There were no significant differences between Negro and Caucasian control patients in any of the variables studied. Total and active rosette formation and rosette inhibition in patients with recurrent genital herpes appeared grossly normal. Both total and early rosettes were significantly depressed in patients with CIS. However, early rosettes were also significantly lower than controls in women with severe cervical dysplasia.

Rosette inhibition titers in patients with cervical dysplasia and CIS are compared with those of control women in Chart 1. There was a clear distinction between patients with severe dysplasia or CIS and controls; however, inhibition titers of patients with mild or moderate dysplasia showed some overlap with the control group. In these normal women there was a significant (p < 0.05) negative

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**Table 1**

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. studied</th>
<th>Median age</th>
<th>Early rosettes</th>
<th>Total rosettes</th>
<th>Inhibition titers*</th>
<th>EAC rosettes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>32</td>
<td>25.3 ± 0.7†</td>
<td>69.0 ± 0.9 †</td>
<td>259 ± 40</td>
<td>26.2 ± 0.4</td>
</tr>
<tr>
<td>Recurrent genital herpes</td>
<td>11</td>
<td>29</td>
<td>25.1 ± 1.6</td>
<td>67.5 ± 1.6</td>
<td>279 ± 71</td>
<td>23.8 ± 0.6</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>3</td>
<td>26</td>
<td>21.8 ± 0.9</td>
<td>66.7 ± 3.5</td>
<td>452 ± 204</td>
<td>24.7 ± 0.4</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>9</td>
<td>23</td>
<td>20.6 ± 1.4</td>
<td>66.7 ± 1.8</td>
<td>1715 ± 472</td>
<td>26.6 ± 1.2</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>11</td>
<td>24</td>
<td>16.4 ± 1.9†</td>
<td>66.1 ± 1.3</td>
<td>4296 ± 711</td>
<td>25.7 ± 1.1</td>
</tr>
<tr>
<td>CIS</td>
<td>10</td>
<td>36</td>
<td>16.2 ± 2.1†</td>
<td>61.0 ± 0.9†</td>
<td>4919 ± 724</td>
<td>24.3 ± 0.8</td>
</tr>
</tbody>
</table>

* Reciprocal of titer giving 25% inhibition of total rosettes; the statistical significance of various comparisons in this group is discussed in the text.
* Rosettes of SRBC coated with antibody and complement.
† Mean ± S.E.
‡ The statistical significance compared with normal controls (p < 0.01).
§ The statistical significance compared with normal controls (p < 0.001).
correlation between the age of the individual and the susceptibility of RFC to inhibition by ALS (Chart 2), necessitating a correction for age during statistical comparisons between patients and controls. Thus, a regression equation relating log of the inhibition titer to age was calculated (\( y = 6.08 - 0.017x \)), and 95% confidence intervals were established for ages corresponding to those of individual patients. None of the patients with recurrent genital herpes or mild dysplasia were significantly different from controls. However, inhibition titers of 6 of the 9 women with moderate dysplasia and all of the 21 patients with either severe dysplasia or CIS were significantly higher than were those of controls.

Inhibition titers in women with moderate and severe cervical dysplasia and CIS showed a significant negative correlation (\( p < 0.05 \)) with the percentage of early rosettes (Chart 3). No such correlation was found in control women.

**DISCUSSION**

The immunocompetence of patients with cervical CIS, as measured by total rosettes, has previously been shown to be depressed (1), and the present study confirms our earlier findings. Rosette inhibition titers were increased in most patients with moderate dysplasia and in all patients with either severe dysplasia or CIS.

Although a previous study (1) had shown an increase in active rosettes in CIS, the present data indicate that early RFC are significantly depressed at this stage of the disease. This discrepancy may be due to the difference in methodology between the 2 studies. The former used a higher SRBC:lymphocyte ratio for detection of active rosettes than did the latter, and it has recently been demonstrated that the higher the SRBC:lymphocyte ratio used, the higher the percentage of rosettes detected (5). In a separate study we found that lymphocytes enriched in early RFC were more susceptible to inhibition by ALS than were unfractionated cells.

Furthermore, when rosettes were formed at ratios of 4:1, 8:1, and 40:1 and the RFC were separated by sedimentation on Ficoll-Hypaque gradients, we found that the lower the ratios used for separation, the higher was the proportion of early RFC in the pellet, and the more susceptible were these cells to rosette inhibition by ALS. These observations suggest that there may be a gradation within the RFC population in susceptibility to ALS. The negative correlation between rosette inhibition titers and age found in normal individuals may thus reflect a gradual alteration in the balance between subsets of lymphocytes with varying degrees of susceptibility to ALS.

In women with the more severe forms of cervical dysplasia and those with CIS, the lymphocyte populations may be altered in such a way that RFC that are extremely susceptible to inhibition increase in frequency, whereas those that are more resistant decrease in frequency. However, such an increase in susceptibility to inhibition would be expected to be associated with an increase in early RFC in these patients. The observed negative correlation between rosette inhibition titers and early RFC (Chart 3) could be reconciled with the above postulate, if it could be shown that rosettes detected at SRBC:lymphocyte ratios lower than 8:1 increased in frequency in these patient groups. An alternative explanation for our observations might be that serum from patients with cervical dysplasia or CIS would block or otherwise render inaccessible some of the SRBC binding sites on the lymphocyte membrane. Such a factor has recently been shown to be present in the serum during pregnancy (10). In current experiments attempts to differentiate between these alternatives are being made.

Although the rosette inhibition test has been shown to

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* R. B. Ashman, S. Sawanobori, M. F. LaVia, and A. J. Nahmias. Inhibition of Early and Late Rosettes by Antilymphocyte Serum, submitted for publication.
discriminate between benign and malignant lesions of the lung (7), only prospective studies will allow us to determine whether inhibition titers have value either as predictors of dysplastic lesions that will progress to cancer or as a prognostic indicator after therapy. Our data do suggest, however, that the rosette inhibition test can detect abnormalities in the thymus-dependent lymphocyte population associated with early events in the neoplastic process. If this test can be generalized to other cancers, it may prove to be of value in detecting, in a precancerous state, patients at risk for cancer.

REFERENCES

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