High-Affinity Erythrocyte Rosette Formation and Inhibition in Premalignant Disease of the Uterine Cervix

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ABSTRACT

Total lymphocyte counts, total rosette formation, high-affinity rosette formation, and the rosette inhibition titer were studied in 104 patients with dysplasia, carcinoma in situ, or Stage I squamous cell carcinoma of the uterine cervix. Although no changes were detected in total lymphocytes or total rosette formation, a significant decrease ($p < 0.001$) was observed in high-affinity rosette formation in patients with moderate and severe dysplasia and carcinoma in situ. The rosette inhibition titer was significantly increased ($p < 0.001$) in all stages of dysplasia and carcinoma in situ; incubation of patients' serum with normal lymphocytes did not reproduce this change but significantly decreased the rosette inhibition titer of the patients' lymphocytes ($p < 0.001$). No change in high-affinity rosette formation or the rosette inhibition titer was observed in patients with Stage I squamous cell carcinoma as compared to controls. These changes are postulated to represent alterations in the normal balance of T-cell subpopulations during the early stages of tumor development.

INTRODUCTION

Premalignant tissues express antigens which are cross-reactive with tumor-associated antigens of the corresponding malignant tumor (1, 18, 31, 35), but the kinetics of the human immune response to the expression of neoantigens has not yet been defined. Stage-related immune suppression is well recognized in established cancer (32). However, immune suppression, or stimulation, has not generally been demonstrable in the precursor phases of human cancer.

Sawanobori et al. (29) demonstrated changes in total and active rosette formation and the RIT in dysplasia and CIS of the uterine cervix; these changes were postulated to represent suppression of cellular immune competence. Total rosette formation represents the total T-cell count, while active rosette formation defines a functional subset of T-cells characterized by high-affinity receptors for SRBC (21, 28). Production of leukocyte migration-inhibitory factor in response to mitogen or specific antigen stimulation appears to be restricted to those T-cells capable of active rosette formation (27). These subpopulations of T-cells may provide an index of cellular immune status in cancer patients (36, 37).

Bach and Antoine (2) described the inhibition of rosette formation by prior incubation of lymphocytes with subcytotoxic dilutions of heterologous ALS. Clinical and animal studies indicated that changes in rosette inhibition reflected stimulation and suppression of the cellular immune system (3, 8, 17). Such changes may be associated with disturbance of T-cell subset populations (13) or the presence of serum factor binding to lymphocyte surface antigens (15).

Dysplasia and CIS of the uterine cervix provide an excellent model for the study of human premalignant disease. We set out to confirm and extend the work of Sawanobori et al. (29) by exploring the hypothesis that disturbances of cellular immune competence occur in premalignant disease. Total lymphocyte counts, total rosette formation, high-affinity rosette formation (34), and the RIT were studied in patients with dysplasia, CIS, and Stage I SCC of the cervix. The RITs of normal lymphocytes exposed to patients' serum were studied to determine whether the observed changes in RITs reflected binding of serum factors to lymphocytes or reflected intrinsic changes of lymphocyte subpopulations.

MATERIALS AND METHODS

The study population comprised 104 women referred for colposcopic examination with a recent history of one or more abnormal Papanicolaou smears. Blood was collected before commencement of treatment, and assays were performed without prior knowledge of the ultimate histology. Definitive histological diagnosis was provided in each case by colposcope-directed biopsy. Women with conditions known to be associated with disturbances of the cellular immune system, such as acute viral syndromes, coexistent cancer, and autoimmune diseases, and patients on immunosuppressive chemotherapy were excluded. On the basis of normal clinical and/or histological findings and normal follow-up smears, 22 women were deemed controls.

Lymphocytes were isolated from 20 ml of fresh heparinized venous blood by centrifugation over a Ficoll-Paque gradient (5) and collection of the interface layer between the Ficoll-Paque medium and plasma. This suspension was washed in balanced salt solution 3 times before ultimate resuspension in HBSS at a concentration of $4 \times 10^7$ cells/ml.

Fresh SRBC were obtained weekly from a single sheep throughout the course of these experiments and stored as heparinized whole blood at $4^\circ$. Before use, 0.1 ml of whole blood was resuspended in 5.0 ml of HBSS and washed 3 times in this medium before resuspension at the appropriate concentration in HBSS.

Total and differential WBC were performed by standard laboratory methods. High-affinity rosette formation was determined by the methods of West et al. (34). Lymphocyte suspension (0.1 ml) was mixed with 0.2 ml of fetal calf serum (heat inactivated, adsorbed with SRBC, and stored as 4.0-ml aliquots for the duration of these experiments). SRBC suspension (0.2 ml) at a concentration of $8 \times 10^7$ cells/ml was added to a final SRBC:lymphocyte ratio of 40:1. After centrifugation at 100 g for 5 min, the cell pellet was incubated for 1 to 12 hr at $29^\circ$ in a water bath. Prior to resuspension, 0.2 ml of supernatant was removed to facilitate counting. After resuspension by gentle tilting, the proportion of rosette-forming mononuclear cells was determined by counting at least 200 cells in a hemocytometer chamber. A rosette-forming cell was defined as a lymphocyte with 3 or more adherent SRBC, and the results were expressed as the percentage of rosette-forming cells. Each test was performed in duplicate, and the mean and S.D. were calculated.

Total rosette formation was determined as above except that the SRBC:lymphocyte ratio was increased to 120:1 (concentration of SRBC...
suspension, $2.4 \times 10^8$ cells/ml, and the cell pellet was incubated for 1 to 24 hr at $4^\circ$ before resuspension and counting. The total lymphocyte counts, total rosette counts, and high-affinity rosette counts were statistically analyzed using the Mann-Whitney test for nonparametric data.

The RIT was based on the methods of Morton et al. (15) with modifications to facilitate several simultaneous assays. A single batch of lyophilized horse anti-human lymphocyte serum donated by Dr. K. James, Department of Surgery, University of Edinburgh, was reconstituted and stored in 0.1-ml aliquots at $-30^\circ$ for use in these experiments. Prior to use, 0.1 ml of ALS was thawed and diluted in 10 ml of HBSS and further serially diluted to 1:128 $\times 10^5$ parts. One-tenth ml of lymphocyte suspension was added to 0.1 ml of each ALS dilution, and all tubes were then incubated at $37^\circ$ for 30 min together with 2 control tubes, each containing 0.1 ml of lymphocyte suspension and 0.1 ml of HBSS. After incubation, 0.2 ml of fetal calf serum and 0.1 ml of SRBC suspension ($1.6 \times 10^6$ cells/ml) were added to each tube and centrifuged at $1000 \times g$ for 5 min. The cell pellets were incubated at $4^\circ$ for 1 to 12 hr before resuspension and counting as described previously. The percentage of mean control rosette formation at each ALS dilution was determined, and the RIT was calculated graphically as the reciprocal of the dilution producing 25% inhibition of control rosette formation.

The effect of patients' serum on the RIT of normal lymphocytes was determined by preincubation of 0.4 ml of control lymphocyte suspension ($1.6 \times 10^6$ cells/ml) from normal healthy donors with 1.6 ml of heat-inactivated patients' serum for 30 min at $37^\circ$. After incubation, 3.0 ml of HBSS were added, and the suspension was centrifuged at $280 \times g$ for 10 min. The cells were then resuspended in 1.6 ml of HBSS, and the RIT was determined as described above. The RITs in this group, and those observed with patients' lymphocytes, were converted to natural logarithms for calculation of means and S.D. and for statistical analysis using Student's t test.

**RESULTS**

The distribution and mean age of each patient group are shown in Table 1. All preinvasive histological stages were well matched for age with the control group. As would be expected from the known biological behavior of cervical SCC, patients with invasive lesions were significantly older than those who were considered to have precursor lesions (Student's t test, $p < 0.05$). No significant alteration was seen in the total lymphocyte count or total rosette formation in patients with dysplasia, CIS, or Stage I SCC of the cervix (Table 1).

High-affinity rosette formation in moderate and severe dysplasia and CIS was significantly depressed, compared to control values (Mann-Whitney test; $p < 0.001$). In patients with mild dysplasia, the values appeared to have a bimodal distribution around control values and values seen in patients with more severe lesions. Significant depression compared to controls was not seen in Stage I SCC, and the results were significantly greater than in CIS, the immediate precursor of Stage I SCC (Table 1).

In Table 2, the RITs in each group are shown. Increases in the RIT were observed with increasing degrees of cervical epithelial atypia. These changes were highly significant in all stages of dysplasia and CIS when compared to control values. Three of 62 patients with moderate and severe dysplasia and CIS demonstrated RITs below an arbitrary value of $8 \times 10^5$. These 3 patients also had high-affinity rosette formation within 1 S.D. of the control mean. One of 21 controls had a raised RIT, and this control also showed high-affinity rosette formation outside the control range. As with high-affinity rosette formation, patients with mild dysplasia showed a wide range of RIT values. Five of 6 patients (corresponding to those in the high-affinity rosette formation study) with Stage I SCC had titers within the normal range. There was a significant negative correlation ($n = 100; r = 0.3031; p < 0.01$) when the RIT was plotted against high-affinity rosette formation in each patient and control (Spearman's rank coefficient correlation). No correlation between the RIT and age, as described by Sawanobori et al. (28), was established in this study population.

When normal lymphocytes were incubated with sera from patients with dysplasia and CIS, no increase in the RIT was demonstrated (Table 3). In fact, a significant decrease, compared to control, was seen ($p < 0.001$). This decrease, which was consistently on one serial dilution, was not observed when control sera were used. Changes in the concentration of test serum (in HBSS), decreases in the incubation temperature (to $4^\circ$), and

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**Table 1**

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No.</th>
<th>Age (yr)</th>
<th>Total lymphocytes ($\times 10^8$)</th>
<th>% of total rosettes</th>
<th>% of high-affinity rosettes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22</td>
<td>29.0 ± 8.8$^a$</td>
<td>2.1 ± 0.7</td>
<td>71.44 ± 3.82</td>
<td>49.99 ± 3.25</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>11</td>
<td>35.2 ± 11.8</td>
<td>2.6 ± 0.9$^a$</td>
<td>69.81 ± 3.61$^b$</td>
<td>45.34 ± 4.76$^c$</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>21</td>
<td>30.4 ± 12.4</td>
<td>2.3 ± 0.6$^c$</td>
<td>70.75 ± 3.53$^b$</td>
<td>39.35 ± 7.83$^c$</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>24</td>
<td>26.7 ± 5.6</td>
<td>2.6 ± 0.8$^a$</td>
<td>69.91 ± 4.52$^b$</td>
<td>40.85 ± 5.04$^d$</td>
</tr>
<tr>
<td>CIS</td>
<td>19</td>
<td>33.7 ± 12.8</td>
<td>2.4 ± 0.8$^a$</td>
<td>68.98 ± 4.22$^b$</td>
<td>40.82 ± 1.13$^b$</td>
</tr>
<tr>
<td>Stage IA SCC</td>
<td>5</td>
<td>40.6 ± 10.9</td>
<td>2.0 ± 0.8$^a$</td>
<td>70.11 ± 4.12$^b$</td>
<td>50.97 ± 4.79$^b$</td>
</tr>
</tbody>
</table>

*$^a$ Mean ± S.D.
*$^b$ Not significant Mann-Whitney test.
*$^c$ $p < 0.02$ (Mann-Whitney test).
*$^d$ $p < 0.001$ (Mann-Whitney test).

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**Table 2**

<table>
<thead>
<tr>
<th>Rosette inhibition titers</th>
<th>n</th>
<th>RIT (natural logarithm $\times 10^8$)</th>
<th>$p^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21</td>
<td>7.88 ± 0.69$^b$</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>11</td>
<td>8.90 ± 0.70</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>21</td>
<td>10.07 ± 0.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>23</td>
<td>10.25 ± 0.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CIS</td>
<td>18</td>
<td>10.84 ± 0.56</td>
<td>NS$^c$</td>
</tr>
<tr>
<td>Stage IA SCC</td>
<td>6</td>
<td>8.41 ± 1.16</td>
<td></td>
</tr>
</tbody>
</table>

*$^a$ Statistical comparison of each group with control by Student's t test.
*$^b$ Mean ± S.D.
*$^c$ NS, not significant.

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**Table 3**

<table>
<thead>
<tr>
<th>Effects of incubation with serum on the RIT of normal lymphocytes</th>
<th>n</th>
<th>RIT ($\times 10^8$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>7.685 ± 0.203$^a$</td>
</tr>
<tr>
<td>Patient's sera</td>
<td>10</td>
<td>7.247 ± 0.203 $^a$</td>
</tr>
<tr>
<td>Control sera</td>
<td>2</td>
<td>7.695 ± 0.064 $^a$</td>
</tr>
</tbody>
</table>

*$^a$ Mean ± S.D.
increases in the length of incubation (to 4 hr) did not alter these findings. There was no relationship between the degree of the serum-mediated decrease of the RIT and the titer observed with the patients' own lymphocytes.

DISCUSSION

The total lymphocyte count and the proportional T-cell count, as assessed by total rosette formation, were not altered in dysplasia, CIS, and Stage I SCC of the cervix. This is in accord with earlier studies (26) and is consistent with the general observation that depression of these parameters is observed only with advanced cancer (14). However, Sawanobori et al. (29) observed that significant depression of total rosettes in CIS and variations in technique probably account for this inconsistency. Our preliminary experiments established that a SRBC:lymphocyte ratio of 40:1, as used in the study of Sawanobori et al., was suboptimal for rosette formation. These conditions may therefore have preferentially selected populations of high-affinity rosette-forming cells rather than the total population of rosette-forming cells.

The terms "active" and "early" T-cell rosettes are usually used synonymously to refer to cells identified after a short period of incubation at 37° (36-38). The 29° high-affinity rosette assay (34), as used in the present study, utilizes a different laboratory technique and yet may estimate a similar subset of T-cells. Our finding, that it provided excellent discrimination between patients with cervical dysplasia and CIS and controls, is in accord with other studies describing the utility of this assay in identifying patients with early cancer (3, 6). The assay also compares well with established techniques in the assessment of cellular immune competence in cancer patients (19, 24), although the cellular and functional characteristics of this T-cell subpopulation remain to be defined. Whether the observed changes represented intrinsic disturbances of the normal balance of T-cell subpopulations or the blocking of SRBC binding by serum factors was considered in a subsequent study (4).

This study has confirmed the work of Sawanobori et al. (29), who initially described changes in the RIT in cervical dysplasia and CIS. This larger series of patients has indicated that these changes of cellular immune competence are sufficiently characteristic of the premalignant stages of this tumor to provide a parameter of potential diagnostic usefulness. Although the technical difficulties involved with the preparation of an active ALS and the manual counting of cells preclude the use of the RIT as a routine screening test, the results are theoretically important. The significant correlation between the RIT and high-affinity rosette formation perhaps indicates that these assays reflect different aspects of the same basic phenomenon. Further understanding of this immune alteration may provide a suitable marker for the presence of lesions liable to progress to cancer.

Substantial indirect evidence indicates that changes in the RIT reflect changes in immune status. Animal studies indicate that maturation of T-cell lines towards ultimate effector cells is associated with increased resistance to ALS-mediated rosette inhibition, i.e., decreased RIT (25). In vivo stimulation of the human cellular immune system is associated with decreases in the RIT (8). Conversely, artificially induced immunosuppression in animals (3) and humans (17) is associated with increases in the RIT. Human cancers, demonstrated to be associated with nonspecific immunosuppression by delayed hypersensitivity tests, are associated with increased RITs (12, 13). Increases in the RIT are seen in the first trimester of human pregnancy (15) and are mediated by a non-species-specific serum factor which suppresses the cellular immune response in adoptive transfer experiments (16). This factor is postulated to compete with antilymphocyte antibodies at the appropriate surface receptors or sterically block SRBC receptor sites.

Unlike the increases in the RIT in early pregnancy, those in cervical dysplasia and CIS were not mediated by serum factors, and an intrinsic disturbance of T-cell populations must be postulated to explain these findings. The rosette-inhibiting capability of ALS is mediated by antibodies directed towards T-lymphocyte-specific antigens (23, 30), and the RIT has been postulated to quantitate the average expression of these antigens by T-cell populations (13). However, it is known that antibodies within ALS are directed towards at least 2 T-lymphocyte surface antigens: one trypsin resistant and the receptor for cytotoxic antibodies; and the other a spatially distinct trypsin-sensitive receptor corresponding to or associated with the SRBC receptor (10, 11, 21). Expression of the latter antigen, demonstrated to have a molecular weight of approximately 65,000 (22), is increased in phytohemagglutinin-stimulated lymphocytes (20, 37) and in lymphocyte populations stimulated in vitro by antibodies (9). Phytohemagglutinin-stimulated lymphocytes show decreased sensitivity to rosette inhibition by ALS (7). These studies suggest that the RIT provides a quantitative index of the expression of the SRBC receptor complex by T-cell subsets. Because the functional maturation of T-cell lines is associated with characteristic surface antigen expression (33), the average expression of differentiation antigens should provide an index of the functional status of T-cell populations. Although the functional significance of the expression of the SRBC receptor complex remains undefined, murine and human studies and surface receptor studies indicate that increases in the RIT reflect impairment of immune responsiveness. Such changes are seen in patients with cervical dysplasia and CIS and appear to reflect intrinsic disturbances in the balance of T-cell subsets in response to unknown stimuli.

The present study showed, unexpectedly, that incubation of normal lymphocytes with serum from patients with dysplasia and CIS was associated with a decrease in the RIT. As an increased RIT was seen with patients' lymphocytes, a serum-mediated influence must exist to produce an apparent decrease in the sensitivity of the T-cell population to ALS. The short incubation period to produce this change is evidence against induction of changes in the intrinsic balance of T-cell subsets by the serum factor.

Possibly, such a factor may bind to other T-cell surface receptors inducing changes in membrane organization which result in expression of more SRBC binding sites or which separate this site from the ALS binding site. The latter is unlikely in view of experiments describing the close association or correspondence between the ALS and SRBC binding sites (10, 11, 21). The observed decrease in high-affinity rosette formation is strong evidence against the former hypothesis, and subsequent experiments (4) have shown that this decrease is mediated by a serum factor in patients with cervical dysplasia and CIS. Complete blockage of rosette formation in a subpopulation of T-cells by this factor would allow measurement of the RIT of only the remaining lymphocytes, of which the average expression of T-surface antigens may be different from that of the whole T-cell population. Binding of ALS to blocked cells may also decrease...
the effective concentration of ALS, i.e., increase the RIT as observed. Clarification of these possibilities must await fractionation and characterization of the surface characteristics of the relevant T-cell subpopulations.

An unexpected finding was the apparent reversion to normal immune competence in 5 of 6 patients with Stage I SCC of the cervix. Histologically, mononuclear cell infiltrates were seen surrounding areas of malignant invasion. These were not present in CIS or in the one patient with Stage I SCC who demonstrated immunosuppression. It appeared that invasion or morphological changes were associated with a further alteration in the interaction between the developing tumor and the immune system. Similar histological appearances have also been observed in patients with colon adenomata who show positive tube leukocyte adherence inhibition responses (1). Whether the result of the apparently delicate balance between immune suppression and immune stimulation in patients with premalignant conditions influences the course of tumor development remains to be determined.

In summary, suppression of cellular immune competence has been demonstrated in patients with dysplasia and CIS of the uterine cervix. Changes in the RIT represent intrinsic disturbances in T-cell differentiation. Elucidation of the mechanism of these disturbances may provide information of value both in the diagnosis and treatment of premalignant and early malignant lesions.

REFERENCES

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