Depression of High-Affinity Rosette Formation in Dysplasia and Carcinoma in Situ of the Uterine Cervix: Mediation by Serum Factors

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ABSTRACT

High-affinity erythrocyte rosettes (HA-RFC) have been shown previously to be depressed in patients with dysplasia and carcinoma in situ of the uterine cervix. The effect of serum from these patients on HA-RFC in normal lymphocytes was studied to further elucidate the nature of immunological changes in early cancer. Sera from patients with dysplasia and carcinoma in situ significantly decreased HA-RFC in normal lymphocytes as compared to control serum ($p < 0.001$). Serum inhibitory activity correlated well ($p < 0.001$) with HA-RFC in the patients' lymphocytes and was postulated to represent the presence of a serum factor in these patients. Liquid chromatography studies showed this factor to have a molecular weight of approximately 50,000. Additional factors with molecular weights of 73,000 and 88,000 were present in patients with carcinoma in situ but not in patients with dysplasia. Serum inhibitory activity was not observed in Stage I squamous cell carcinoma. These factors may represent antigen-specific suppressor factors produced in response to the expression of neoa antigens by premalignant cells and may be involved in "sneaking through" of developing tumors.

INTRODUCTION

Subpopulations of T-cells, characterized by a high avidity for SRBC, can be identified under experimental conditions which are suboptimal for erythrocyte rosette formation (31, 34). The "active" rosette assay (34) and the 29° high-affinity rosette assay (31) appear to provide a more accurate index of cellular immune function than total T-cell counts. Active rosette-forming cells are characterized by distinctive biochemical characteristics of the cell membrane (37) and increase in numbers in subjects with effective delayed hypersensitivity reactions to intracutaneous antigen injections (9). Increases in active rosettes appear to be mediated by lymphokines elaborated by sensitized cells in reaction to antigen exposure (1) and by similar factors in mixed lymphocyte reactions (36). Depression of active rosettes is demonstrated in patients with hereditary immunodeficiency states (34) and in patients with cancer (27, 28, 35).

HA-RFCs, which are formed at 29°, appear to provide a more accurate discrimination of the presence of cancer (8, 23) than active rosette assays. These T-cells have a characteristic anatomical distribution (30) and are characterized by the absence of surface Fc receptors. "Low-affinity" T-cells have Fc receptors and appear to be responsible for a significant component of in vitro "natural killer" activity (14, 29).

Consideration of the experimental conditions of the active and high-affinity rosette assays suggests that each assay probably defines a similar population of T-cells (33), although accurate definition of human T-cell populations is required to confirm this. We have demonstrated previously (2) in patients with dysplasia and CIS of the uterine cervix that increases in the RIT reflected intrinsic disturbances in the differentiation of T-cell surface antigens; indirect evidence supported the view that these changes represented immunosuppression. We also demonstrated depression of HA-RFC in the patients. This finding may reflect intrinsic disturbances of the normal balance of high- and low-affinity T-cell subsets. Alternatively, there may be serum factors which bind to lymphocyte surface receptors and mask normal proportions of underlying T-cell subsets. The present study was performed to differentiate between these 2 possibilities and to further characterize the immunological changes accompanying premalignant disease.

MATERIALS AND METHODS

The study population comprised 104 women referred for colposcope 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 by colposcope-directed biopsy or cone 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 smear, 22 women were deemed controls. Lymphocytes were isolated from 20 ml of fresh heparinized venous blood by centrifugation over a Ficoll-Paque gradient (3) 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 the appropriate concentration.

Fresh SRBC were obtained weekly from a single sheep throughout the course of these experiments and stored as heparinized whole blood at 4°. 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.

The HA-RFC assay was performed as described by West et al. (29). 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 $\times g$ for 5 min, the cell pellet was incubated for 1 to 12 hr at 29° in a water bath. Prior to resuspension, 0.2 ml of supernatant was removed to facilitate counting. After resuspension by gentle titling, 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. The results were analyzed statistically by the Mann-Whitney test for nonparametric data.

To study the effects of serum from controls and patients with dysplasia, CIS, and Stage I SCC on HA-RFC, lymphocytes were obtained from normal healthy donors and adjusted to a concentration of 8 × 10⁶ cells/ml in HBSS. Sterile serum obtained from clotted blood was heat inactivated and stored at −30° until use. Lymphocyte suspension (0.2 ml) (8 × 10⁶ cells/ml) and 0.2 ml of thawed serum were mixed and incubated at 37° for 30 min. After incubation, 1.0 ml of HBSS was added, and the suspension was centrifuged at 200 × g for 10 min before resuspension in 0.2 ml of HBSS. The concentration of this suspension was determined and adjusted to 4 × 10⁶ cells/ml. Two tubes of lymphocyte suspension were simultaneously run in HBSS as controls. HA-RFC was determined as described above, and the result was expressed as the percentage of inhibition of control rosette formation by serum. These results were statistically analyzed using the Mann-Whitney test for nonparametric data.

For determination of the molecular weight of serum factors mediating changes in HA-RFC, heat-inactivated sera from patients with moderate dysplasia, severe dysplasia, CIS, Stage I SCC of the cervix, and normal cervical histology were analyzed by liquid chromatography. Prior to running samples of the column, serum from each patient was thawed, dialyzed in 1 liter of phosphate-buffered saline (0.5 mM sodium phosphate in 0.9% sodium chloride solution at pH 7.4) at 4° for 6 hr, and then dialyzed in fresh phosphate-buffered saline for a further 6 hr.

Initial fractionation of sera from 2 patients with severe dysplasia was achieved on a Ultragel AcA-34 column. Calibration of this column was achieved by running samples of aldolase (M, 150,000), albumin (M, 67,000), ovalbumin (M, 43,000), and chymotrypsinogen A (M, 25,000) and determining the elution profiles of these proteins by absorbance measurements of 1.0-ml fractions at a wavelength of 280 nm. A standard curve was constructed from this information. Peaks of serum high-affinity rosette inhibition were determined by preincubation of normal lymphocytes with each fraction obtained from the column as described above. Percentage of inhibition was plotted against elution volume, and the molecular weight of peaks of activity was estimated from the standard curve.

The results of the initial study prompted the use of Sephadex G-100 to obtain increased resolution of inhibitory peaks. Fractions (0.5 ml) were obtained from this column which was standardized as described above. The molecular weight of peaks of activity was determined as for the AcA-34 column.

RESULTS

The complete results of the HA-RFC assay in the study population have been described previously (2). In summary, HA-RFCs were decreased in moderate and severe dysplasia and CIS. This decrease was highly statistically significant when each group was compared to control. No change was seen in patients with Stage I SCC. While one of 22 controls demonstrated HA-RFC below 43.5%, 2 S.D.s below the control mean), only 3 of 62 patients with moderate or severe dysplasia or CIS had values above 43.5%. Patients with mild dysplasia demonstrated a wide scatter between control values and values within the range of patients with more advanced atypia.

Sera from 90 subjects in this study was preincubated with normal lymphocytes, and the results are shown in Table 1. Sera from patients with moderate and severe dysplasia and CIS inhibited rosette formation of normal lymphocytes. The mean and S.D. of the results obtained with control sera are consistent with the normal variation of HA-RFC assay and indicate that no inhibition was seen with control sera. The inhibition observed in moderate and severe dysplasia and CIS was statistically significant in each stage when compared to control. Sera from patients with mild dysplasia had a wide range of inhibitory activity, consistent with the wide range of HA-RFC and RITs seen in this group. No serum inhibitory activity was detected in 5 patients with Stage I SCC who had shown normal HA-RFC and RITs. Serum was not available from the sixth patient, the only one with Stage I SCC who had depressed HA-RFC.

The magnitude of the serum inhibitory activity in patients with dysplasia and CIS was comparable to that of the depression in HA-RFC in the patients’ lymphocytes, namely 20%. When the percentage of serum inhibitory activity was plotted against HA-RFC with the patients’ lymphocytes, a highly significant correlation (r = 0.79; p < 0.001) was demonstrated between these 2 parameters.

In 2 patients with severe dysplasia, serum inhibitory activity eluted in a single 1.0-ml fraction on an AcA-34 column corresponding to a molecular weight of approximately 47,500 to 54,500. Variation in the activity of other fractions was consistent with the inherent error in this technique, showed no consistent pattern, and was considered to be random and not due to serum activity.

For all further liquid chromatography studies, Sephadex G-100 gel was used, as it is characterized by maximum resolution at molecular weights of 10,000 to 100,000. No consistent inhibition of rosette formation was seen with fractions from sera of control patients, and observed variation remained within normal limits. When sera from 2 patients with moderate dysplasia were studied, serum inhibitory activity eluted within two 0.5-ml fractions corresponding to a molecular weight of 51,000 (range, M, 48,500 to 54,500). No other inhibitory peaks were seen. When sera from 2 patients with CIS were examined, an inhibitory peak again eluted within two 0.5-ml fractions corresponding to an approximate molecular weight of 51,000. One of the patients with CIS demonstrated an additional peak of inhibitory activity over 2.0 ml, which corresponded to an approximate molecular weight of 51,000. Both patients also showed peaks of activity eluting with 7.5 ml, corresponding to a molecular weight of approximately 88,000. No other consistent peaks of activity were seen in sera from CIS patients. When sera from patients with Stage I SCC were considered, no consistent peaks of activity were seen at any molecular weight. Sera from patients with mild dysplasia were not studied, as inhibitory activity in
these was generally insufficient to be clearly distinguished from random activity.

**DISCUSSION**

This study extends our previous observations of immunological parameters in the preinvasive stages of SCC of the cervix and demonstrates a mechanism for the observed changes in HA-RFC formation in those patients. The HA-RFC assay provided a sensitive index of the presence of premalignant changes in this study population, 90% of patients with moderate and severe dysplasia and CIS having HA-RFC levels below the control range (2 S.D.s below the control mean). An important feature of this study population was the low mean age and associated low incidence of benign medical conditions. Disturbances of T-cell subpopulations in nonmalignant conditions and associated with increasing age (32) may limit the screening value of HA-RFC in older populations where the majority of cancers occur.

Serum from 95% of patients with moderate and severe dysplasia and CIS inhibited normal HA-RFC, whereas control sera showed no such inhibition. The strong statistical correlation between the magnitude of serum inhibition and HA-RFC with the patients’ own lymphocytes supports the hypothesis that serum factors which decrease the affinity of T-lymphocytes for SRBC are present in patients with cervical dysplasia and CIS. These factors appeared to mediate the observed depression in HA-RFC. As this inhibition was complete within 30 min, it was unlikely that these factors stimulated proliferation of T-cell subsets. It was felt that this phenomenon represented the binding of serum factors to the lymphocyte SRBC receptor or adjacent surface receptor, or the rearrangement of cell membrane components subsequent to binding to a receptor not associated with the SRBC receptor. Preliminary observations of inhibition at 4°, when membrane fluidity is greatly reduced (32), make the latter alternative less likely.

The assay for serum inhibitory factors was superior to the HA-RFC assay in the definition of patients with cervical premalignant disease. As results were expressed as a percentage of a control value, slight day-to-day variations in rosetting technique were of lesser significance than in the HA-RFC assay. The use of stored serum was convenient, allowing large numbers of simultaneous tests on single days.

The magnitude of depression, by serum factors, of normal rosette formation was constant and consistent with the magnitude of depression of the patients’ own HA-RFC. This suggested that a specific subpopulation of T-cells bound rosette-inhibitory factors. Serum factors identified in breast cancer appear to bind a similar proportion of T-cells (22, 35), and in the latter study, sequential incubations could not increase the magnitude of the inhibition, supporting the hypothesis that, in breast cancer, serum factors bind a specific T-cell subpopulation. Further indirect evidence for this hypothesis in cervical dysplasia and CIS was suggested by our earlier unexpected finding that serum from patients with cervical dysplasia and CIS decreases the RIT of normal lymphocytes. If these serum factors bound across all lymphocyte subpopulations to binding sites near or associated with the SRBC receptor, as do rosette-inhibiting antibodies of ALS (12), an increase in the RIT of normal cells would be expected after incubation with patients’ serum. Complete “blockage” of a subpopulation of T-cells by serum factors would allow the measurement of the RIT of only the remaining lymphocytes, of which the average expression of T-surface antigens may be more or less than that of the complete T-cell population. Binding of ALS to blocked cells could still occur and decrease the effective concentration of ALS measured by the RIT. Direct evidence of the binding of serum factors to specific subpopulations must await fractionation and characterization of the relevant T-cell subpopulation.

Although rosette-inhibitory factors in cervical dysplasia and CIS clearly bind to allogenic lymphocytes, their structure and function remain undetermined. Indirect evidence indicates that decreases in HA-RFC reflect impaired cellular immune competence, but no immunosuppressive role has been demonstrated for factors which inhibit rosette formation. Nevertheless, many different groups of factors which suppress in vitro immune reactions have been described as binding to lymphocytes of humans and animals with tumors (16). Fractionation of serum factors in our patients allowed firm conclusions to be drawn as to their nature. As the primary band of activity is well concentrated within a molecular weight range of 48,000 to 54,000, this serum factor is neither an antigen:antibody complex nor an immunoglobulin. The molecular weight and short incubation period before maximum inhibition is attained are evidence against viral-associated lipoproteins as rosette-inhibitory factors (7). Non-specific products of tissue breakdown (32) would exhibit a wide range of molecular weights and would not be a prominent feature of this small intraepithelial lesion. Soluble tumor antigens are known to be immunosuppressive in vitro but demonstrate a wide range of molecular weights (25). Significant serum levels would not be expected with the small tumor load of cervical CIS and would be expected to increase, rather than disappear, with tumor invasion.

Antigen-specific suppressor factors, coded by the I-J subregion of the H-2 locus in the mouse, have been described with a molecular weight of 50,000 (4). These factors, under different in vitro conditions, may be genetically restricted or nonrestricted (5). I-J-linked factors mediate antigen-specific suppression of lymphokine production in animal tumors (19). In humans, the immune response may be inhibited by different stages of T- and B-cell differentiation, such as by the secretion of humoral suppressor factors which inhibit T-helper activity (29), B-cell immunoglobulin biosynthesis (10), and antigen-stimulated T-cell proliferation (13). Such suppression is genetically nonrestricted (29) and may be antigen specific (5, 18, 26) or nonspecific (10, 13).

The serum factor with a molecular weight of 50,000 in cervical dysplasia and CIS binds to a subpopulation of T-cells, behaves as a discrete substance on a Sephadex column, and is not an immunoglobulin, tumor antigen, or antigen:antibody complex. It is reasonable to speculate that it represents a suppressor factor. Changes in the RIT in these patients have been demonstrated previously to represent an intrinsic alteration of T-cell differentiation (2). Changes in the RIT and HA-RFC in patients with cervical dysplasia and CIS may represent suppressor cell action at different stages of the T-cell response. As generalized suppression of cellular immune competence is not a feature of the premalignant stages of cervical carcinoma (17), our observations may represent suppression which is specific for tumor antigens expressed by dysplastic cells.

Carcinogen-altered cells in the premalignant stage of murine tumor development stimulate the production, by T-cells, of I-J-linked antigen-specific suppressor factors (24). Antigen-specific
immune suppression has also been demonstrated in murine tumors where small inocula of tumor cells were introduced (20), and such suppression appears to be mediated by T-cells (11). Large doses of tumor cells abrogated this suppression. Classical low zone tolerance (21) may be mediated by T-suppressor cells (6). There is a clear parallel between these situations and cervical CIS, where a very small tumor antigen load is presented to the immune system. Antigen-specific immune suppression in cervical dysplasia and CIS may represent an example of sneaking through immunological barriers.

The apparent disappearance of serum inhibitory factors in Stage I SCC of the cervix parallels the reversion to normal RITs in these patients and may represent the abrogation of sneaking through described in animal systems. Such a response to the increasing tumor load would be predicted by the classical tolerance model (21).

Peaks of rosette-inhibitory activity were demonstrated at molecular weights of 73,000 and 88,000 in the patients studied with CIS. Morphological progression from dysplasia to CIS appears to be associated with the expression of 2 tumor-associated antigens detectable in cell membrane extracts (15). These new peaks of rosette-inhibitory activity may represent tumor-associated antigens or suppressor factors of a different activity to the M, 50,000 factor.

In summary, changes in HA-RFC in patients with dysplasia and CIS of the cervix are mediated by serum factors. Changes in HA-RFC and the RIT may represent antigen-specific immune suppression mediated by suppressor cells. Such sneaking through immune barriers may represent one method of immune protection of human tumors at the preinvasive stage.

REFERENCES


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