Flow Cytofluorometric Detection of Tumor-specific Rosette-forming Cells in Patients with Squamous Cell Carcinoma of the Head and Neck

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ABSTRACT

Tumor-specific rosette-forming cells reactive to solubilized tumor antigens conjugated to autologous erythrocytes were quantitated by flow cytofluorometry. Leukocytes from a high frequency of the patients (>70%) with squamous cell carcinoma of the head and neck (SQCC) formed rosettes to the conjugated SQCC tumor antigens but not to other histologically distinct tumor antigens (melanoma and colon carcinoma). Healthy control subjects or tumor patients with other cancers were mostly unreactive to the SQCC tumor extract [1 of 21 (5%) and 1 of 14 (7%) for controls and tumor patients, respectively]. Rosette-forming activity was observed in SQCC patients with primary cancers [22 of 30 (73%)] or in remission [5 of 6 (83%)], whereas patients with tumor recurrence were uniformly unresponsive [0 of 9 (0%)]. Tumor-specific rosette formation was mediated predominantly by monocytes, as identified by histochemical techniques and physiological properties. Rosette formation in reactive patients was abrogated by short-term culture, but the abated response could be restored by incubation with autologous serum or sera from other rosette-forming cell-positive patients. However, responsiveness of nonreactive patients with SQCC recurrence could not be constituted by rosette-forming cell-positive sera. These observations suggested the presence of tumor-reactive monocytes in a high frequency of patients with primary cancer or in remission but not in patients with recurrent disease.

INTRODUCTION

Immune responses of tumor patients with SQCC have been difficult to evaluate in the past. These patients frequently exhibited impaired T-cell function (1, 9, 25) and poor tumor-specific responses assessed by delayed cutaneous hypersensitivity (3, 25), leukocyte adherence inhibition (3, 22), lymphocyte proliferation (24), or other in vitro assays (2, 5). Since reactivity in these assays invariably requires both an immune recognition event and a secondary effector function, anergy among nonresponders can be attributed to deficiencies in either generalized or tumor-specific recognition or effector functions. In this study, we attempted to evaluate the initial recognition events of SQCC patients with an antigen-binding cell assay (23), utilizing tumor antigen-coated autologous erythrocytes.

Antigen-binding cell assays have been applied widely to evaluate conventional antigen recognition (for review, see Ref. 7), but technical difficulties and the use of tumor extracts of questionable specificity have limited their applicability in human tumor immunology (20, 27). In this study, the reactivity of peripheral blood mononuclear cells of cancer patients was evaluated by rosette formation with autologous erythrocytes, which were coated with the relevant, or histologically distinct, 3 mKCl tumor antigen extracts of functional specificity demonstrated previously (3, 22). Frequency analysis of RFC was performed by a flow cytofluorometric technique described previously. This technique provides a more objective approach than does conventional microscopic quantitation by allowing the analysis of multiple replicas and large sampling sizes (10^6 cells/sample) for each antigen. The RFC responses of several patients to different tumor antigen extracts are determined within hours.

We detected a high frequency of tumor-specific RFC response in SQCC patients with primary cancer (>70%) and those in remission (83%). However, SQCC patients with tumor recurrence were uniformly unreactive (0%). This is likely to represent both monocyte and humoral function deficiencies.

MATERIALS AND METHODS

Patients. The study population included hospitalized patients and outpatients at the Portland Veterans Administration Medical Center and University of Oregon Health Sciences Center. Eighty patients (volunteers after informed consent) were evaluated, including 45 SQCC patients (30 patients with primary cancer, 6 patients without evidence of tumor undergoing follow-up therapy, and 9 patients with recurrent tumors); 14 patients with biopsy-proven, newly diagnosed, or persistent non-head and neck cancers or head and neck nonsquamous tumors or tumor free following therapy; and 21 patients without evidence of cancer. Patients that had newly diagnosed SQCC were evaluated before treatment. The returning patient population represented patients in remission and had remained tumor free for at least 30 days. Of the 9 patients studied with SQCC tumor recurrence, 3 had received radiation, surgery, and chemotherapy; 4 had received irradiation and surgery; and 2 patients had received only surgery. Two of these patients were known to be nutritionally depleted. All patients were males ranging from 50 to 70 years of age.

Tumor Antigen Extraction. Tumor tissues were obtained within 4 hr of excision from patients with histologically proven tumors. Tissue was collected from only primary and regionally metastatic lesions from patients for whom surgery was the primary mode of therapy. Normal tissues from these patients were unavailable for control evaluation.

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4 The abbreviations used are: SQCC, squamous cell carcinoma of the head and neck; RFC, rosette-forming cells; TBS, Tris-buffered 0.9% NaCl solution; RPMI 1640, Roswell Park Memorial Institute Tissue Culture Medium 1640; NCS, newborn calf serum.
studies due to human rights considerations. Tissues were finely minced, washed repeatedly with 0.15 M NaCl solution, freeze-dried, and stored at −20° until extraction with 3 M KCl was carried out (15). Briefly, 10⁶ cells were mixed with 15 ml of 3 M KCl (per 1.5 g) overnight at 4° followed by dialysis against water and then 0.9% NaCl solution. After each dialysis, the solution was centrifuged (40,000 × g, 30 min) to remove precipitate. The dialyzed product was then filtered (0.22 μm) and stored at −20°.

RFC Assay. Preparation of donor lymphocytes and antigen conjugation to autologous erythrocytes have been described previously. Briefly, erythrocytes were extracted from whole blood by dextran (6%) sedimentation and reconstituted to 2.5% (v/v) in TBS. Aliquots of 0.1 ml of RBC were added to equal volumes of TBS containing 0.15 to 0.2 mg of the appropriate tumor antigen extract. An equal volume to chromium chloride (0.1%) in TBS was added dropwise and left at 23° (room temperature) for 4 min (6). After appropriate washing, presence of tumor antigens on RBC surfaces was demonstrated separately by using prelabeled ¹²⁵I tumor extracts (data not shown). The antigen-conjugated erythrocytes were reconstituted to 0.25% (v/v) and incubated with equal volumes (at 1 to 2 × 10⁴/ml) of Ficoll:Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.)-extracted mononuclear cells (400 × g, 30 min) from the same donor at 4° for 1 hr (WBC:RBC, 1:10). The samples were acidine orange stained (final concentration, 1 μg/ml) to facilitate enumeration of mononuclear cells during flow cytofluorometry analysis. Although slightly higher levels of RFC can be detected at a higher incubaing RBC concentration (2.5%), this also resulted in high background values from nonspecific cell clumping and electronic coincidence.

Assessment of RFC by Flow Cytofluorometry. RFC frequency analysis by flow cytofluorometry and its correlation with microscopic quantitation have been described previously. Briefly, sample aliquots were subjected to 2-dimensional cytometric analysis (Cytotransferor 4800A; Ortho Instruments, Westwood, Mass.) of green fluorescence (ordinate versus forward-angle light scatter (abscissa)). RFC were detected in a "select count" electronic window precalibrated to exclude >97% of the unrosetted mononuclear cells on the forward-angle light scatter scale (Chart 1). The percentage of RFC response to each tumor antigen was determined with triplicate or quadruplicate samples. Mean RFC response per sample was established with multiple (4 or 5) 200- to 250-μl aliquots (approximately 10,000 nucleated cells). The green fluorescence scale was gated to detect cells with only a single complement of DNA to exclude WBC clumps from the select count enumeration. The total number of nucleated cells in the aliquot (which triggered a green fluorescence signal) was established in the "total count" window. For each donor, specific RFC were determined by the formula % of tumor-specific RFC = % of tumor antigen RFC – % of control antigen RFC

The extensive overlapping in size between specific RFC and background warranted using a subtracted control (instead of an arbitrary size cutoff) in quantitating the frequency of specific rosette formation. Both the Mann-Whitney U and Student’s t statistical analyses were used to establish tumor-specific RFC responses. The RFC response (mean ± S.D.) to each antigen represents the replica mean of triplicate or quadruplicate samples. Differences between the means of the tumor antigen versus control (human serum albumin) rosetting-forming responses were significant if the 2 population samplings did not overlap (Mann-Whitney U, p < 0.05) and had t values significant at 95% confidence levels.

Enrichment of RFC. Since the Cytotransferor 4800A lacks sorting capability, RFC were enriched by gently layering rosetted samples (1 ml WBC incubated with 1 ml antigen-conjugated RBC) over 2 ml Ficoll:Hypaque and centrifuging at 400 × g for 30 min. The RFC-enriched pellet was gently resuspended in 0.25 ml of RPMI 1640, stained for nuclear fluorescence [acridine orange final concentration, 1 μg/ml], or nonspecific esterase (α-naphthyl acetate; Sigma Chemical Co., St. Louis, Mo.), and examined by fluorescent microscopy.

Chart 1. Detection of RFC by flow cytofluorometric analysis. Total count and select count electronic windows were selected on the basis of computer-drawn scattergrams of nuclear fluorescence (ordinate) versus cell size (abscissa). Each point represents an event triggered by a nucleated cell (nuclear green fluorescence) with a forward-angle light scatter signal (in proportion to the cell size). The total count window (Region 1) was selected to enumerate all nucleated cells. Anucleated RBC do not trigger a green fluorescent signal and are not enumerated by electronics. The select count window (Region 2) was selected to exclude >97% of the unrosetted population (abscissa). a, scattergram of mononuclear cells plus unconjugated autologous erythrocytes; select count included less than 3% (2.1%) of the population. b, scattergram of mononuclear cells after incubation with control antigen human serum albumin RBC; select count represented 3.9% of total count. c, scattergram of mononuclear cell from SQCC patient after incubation with SQCC tumor extract; select count represented 5.3% of total count.

Monocyte Depletion. Monocyte depletion by Sephadex G-10 has been described elsewhere. Briefly, mononuclear cells suspended in RPMI 1640:5% NCS were layered on a 5-ml G-10 (Pharmacia) column and incubated at room temperature for 15 min. The column was washed with a 5-fold volume of RPMI 1640:5% NCS and contained cells with less than 1% monocytes (monocyte depleted) by Wright’s staining. In certain experiments, mononuclear cells were separated into T- and B-enriched fractions by sheep RBC E-rosetting or nylon wool column treatment, as described earlier.

Mononuclear Cell Incubation in Vitro. To evaluate the rosetting-forming responses of responder (RFC positive) cell populations after in vitro culture, mononuclear cells were suspended in RPMI 1640:5% NCS and incubated at 37° for 24 hr. The cultured cells were washed twice with serum-free RPMI 1640 and resuspended to the original...
concentration for analysis. Serum from patients was obtained by freeze-thawing whole-blood-derived plasma (250 g, 10 min). Incubation with autologous or allogeneic cells (4 °C, 30 min) was carried out in the ratio of the relative yield during extraction; i.e., if 10 ml of plasma and 2 x 10^6 mononuclear cells were obtained from the whole-blood sample, then 2 ml of serum were incubated with 4 x 10^6 cells. The WBC were then washed, resuspended to the original concentration, and incubated with various antigen-conjugated RBC.

**DEAE: Serum Fractionation.** Immunoglobulin fractions were obtained by 50% (NH₄)₂SO₄ precipitation of pooled sera of responsive patients with primary or recurrent SQCC. The pellet precipitate (800 x g, 30 min) was resuspended in 0.01 M phosphate buffer, dialyzed for 72 hr, filtered to remove aggregates, and then passed over a DEAE-column (Pharmacia) equilibrated previously with 0.01 M phosphate buffer. The IgG-enriched fraction (Fraction I) was collected as the first major peak eluted and characterized by immunoprecipitation in gels (Ouchterloney reaction). Protein retained by DEAE-column was eluted with 0.1 M phosphate buffer as the IgG-depleted fraction (Fraction II).

**Protein retained by DEAE-column was eluted with 0.1 M phosphate buffer as the IgG-depleted fraction (Fraction II).**

**RESULTS**

**Evaluation of Tumor-specific Rosette Formation.** Previously, we demonstrated antigen binding to microbial and protein antigens among immunized donors by rosette formation to antigen-conjugated autologous erythrocytes, using a flow cytfluorometric technique. We have adopted a similar approach to examine tumor-specific recognition responses of SQCC patients (Table 1). Mononuclear cell samples from patient and control groups, including healthy subjects and patients with other cancers, were coded and tested in a blind fashion against a control protein (human serum albumin) and a panel of tumor antigens, including SQCC, colon carcinoma, and melanoma.

The RFC responsiveness of patients with SQCC to the relevant tumor extracts was compared to that of the control patient groups (Chart 2). To evaluate these determinations objectively, only those rosette-forming responses that were significantly higher from the control values (p < 0.05) were considered positive. Thus, patients that exhibited responses to tumor antigens that were elevated but not statistically different from the control values (Chart 2A, •) were still considered nonresponsive.

Among the patient population with SQCC, greater than 70% (22 of 30) with untreated, biopsy-proven, primary cancer (Chart 2A) demonstrated a positive response. Similarly, a high frequency of reactivity (5 of 6 (83%)) was observed among the small number of patients in remission (Chart 2B). However, we did not detect any specific response (0 of 9 (0%)) among patients with tumor recurrence (Chart 2C).

There were significantly lower response frequencies (5 and 7%, respectively) among control patient groups, including healthy donors (Chart 2E) or tumor patients with non-head and neck squamous tumors and head and neck nonsquamous cancers (Chart 2D). Conversely, SQCC patients reacted primarily to the SQCC extract with a low frequency of cross-reactivity to control tumor extracts (Chart 3), such as melanoma (1 of 16 patients) or colon carcinoma (2 of 11 patients). Patients with recurrent SQCC did not react to any of the tumor extracts (data not shown). In a small number of reciprocal determinations, melanoma patients were responsive to the melanoma extract (14 of 33 positive) and negative to the SQCC extract (1 of 33 positive).

**Specificity of RFC Response.** The responsiveness of SQCC patients to the relevant tumor antigen extract, together with the low reactivity of the control patient groups, suggested specificity of rosette formation. The question of specificity was further examined by studying RFC responsiveness before and after a preincubation treatment with either the relevant or control antigen. The RFC response shown in Chart 4 is representative of the observations on 5 SQCC patients evaluated after preincubation treatment with either the relevant or control antigen.

![Chart 2. Rosette-forming response to SQCC conjugated autologous erythrocytes by different patient groups. Each point, a specific RFC response of a patient (after background correction) to SQCC. A, patients with primary cancer of SQCC; B, SQCC patients in remission; C, patients with recurrent cancer of SQCC type; D, non-head and neck squamous and head and neck nonsquamous patients; E, control group with no evidence of malignant disease. O, statistically significant response (Student's t test, p < 0.05); •, response that was not statistically significant (p > 0.05). Tu-Ag, tumor antigen.]

**Table 1**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>% of rosette formation</th>
<th>% of tumor-specific rosettes&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SQCC</td>
</tr>
<tr>
<td>SQCC</td>
<td>2.95 ± 0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.95 ± 0.11</td>
</tr>
<tr>
<td>Melanoma</td>
<td>2.45 ± 0.27</td>
<td>2.60 ± 0.17</td>
</tr>
<tr>
<td>Control</td>
<td>2.82 ± 0.20</td>
<td>2.91 ± 0.35</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of RFC<sub>tumor antigen</sub> - percentage of RFC<sub>human serum albumin</sub>
<sup>b</sup> HSA, human serum albumin; NT, not tested.
<sup>c</sup> Mean ± S.D.
<sup>d</sup> Statistically significant responses (Student's t test, p < 0.05).
<sup>g</sup> Numbers in parentheses, responses not statistically significant (Student's t test, p > 0.05).
cells were incubated for 24 hr, as described above, and tested for their ability to reacquire a tumor-specific rosette-forming response. Cells from 2 patients with recurrent cancer remained unresponsive after overnight culture (Table 2) and did not acquire any reactivity from autologous serum. Sera that had an arm ing ability demonstrated previously were also ineffective. Similarly, cells from another patient with recurrent tumor could not acquire a keyhole limpet hemocyanin-specific RFC response after treatment with keyhole limpet hemocyanin-specific antibodies, which had been shown to be effective in arming a normal cell population. It was apparent that nonresponders lacked the mononuclear subpopulation required to mount a serum-mediated antigen recognition response. All donors that were examined, however, contained a normal level of monocytes (15 to 21%).

Characterization of Serum-arm ing Factor. To identify the mediating factor(s) present in immune sera, we evaluated the effect of anti-human immunoglobulin treatment on the tumor-specific rosette-forming response. As shown in Table 3, responsive donors lost their reactivity following preincubation with anti-human immunoglobulin, suggesting that a majority of specific RFC carried surface immunoglobulins. Similarly, the reacquired SQCC reactivity of cells cultured in vitro was also inhibited by antimunoglobulin treatment (Chart 5).

To further characterize the serum-arm ing elements, we compared incubation with the relevant or control (melanoma) antigen. In all cases, only preincubation with SQCC antigen (but not the control tumor antigen) was effective in inhibiting the RFC response ($p < 0.01$).

Involvement of Monocytes in Tumor-specific RFC. These observations suggest that rosette formation may reflect a specific tumor recognition event. Furthermore, the process appears to be disease stage related, as no reactivity was detected among patients with tumor relapse. To identify the cell type of the RFC, reacted samples were enriched for rosettes by isopyknic centrifugation over Ficoll Hypaque. Microscopic examination of the RFC-enriched samples following $\alpha$-naphthyl acetate esterase or acridine orange staining demonstrated the predominance of monocytes among the tumor-specific rosette-forming population. In 5 separate determinations, a mean value of 90% (90 ± 10% (S.D.)) of RFC appeared to be esterase-positive mononuclear cells with a considerably lower frequency of participating lymphocyte RFC (0 to 20%; mean, 8.6 ± 8.9%). The identification was corroborated with acridine orange-stained samples (85 ± 13% monocytes).

Furthermore, whereas tumor-specific rosette-forming reactivity was not affected by T- or B-cell depletion, monocyte depletion by Sephadex G-10 passage resulted in >95% loss of tumor-specific reactivity in 5 of 5 separate experiments (data not shown).

Mediation of Tumor-specific Rosette Formation by Serum Factors. Since monocytes were not known to generate antigen-specific receptors (8), responsive patient cells were cultured in vitro to determine if tumor-specific receptors were derived endogenously. RFC reactivity was lost after a 24-hr incubation period in 5 of 5 determinations (Chart 5) and did not regenerate after 4 days (data not shown). However, the cultured cells reacquired a tumor-specific RFC response after incubation with autologous serum (Chart 5) or serum from another responder. The reacquired responses approximated, but were never statistically significant ($p > 0.05$).

Monocyte Dysfunction of Recurrent Patients. To establish the nature of unresponsiveness of recurrent patients, cells were incubated for 24 hr, as described above, and tested for their ability to reacquire a tumor-specific rosette-forming response. Chart 3. Tumor-specific rosette-forming response of SQCC patients to a panel of tumor extracts: SQCC; melanoma (MEL); and colon carcinoma (CC). ○, statistically significant response (Student's $t$ test, $p < 0.05$); ●, responses that were not statistically significant ($p > 0.05$). Ag, antigen.
Table 2

Antibody-mediated tumor antigen recognition in patients with SQCC

<table>
<thead>
<tr>
<th>% of antigen-specific rosette formation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SQCC RFC</th>
<th>KLH&lt;sup&gt;b&lt;/sup&gt; RFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 24-hr cell culture&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+ Auto</td>
<td>+ Arming serum&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Donor cell</td>
<td>Untreated</td>
<td>Autologous serum</td>
</tr>
<tr>
<td>Control</td>
<td>−0.20 ± 0.30&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>Primary SQCC</td>
<td>0.73 ± 0.27&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.60 ± 0.37&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Recurrent SQCC</td>
<td>−0.06 ± 0.32</td>
<td>0.09 ± 0.29</td>
</tr>
<tr>
<td>Recurrent SQCC</td>
<td>−0.64 ± 0.32</td>
<td>−0.21 ± 1.22</td>
</tr>
<tr>
<td>Recurrent SQCC</td>
<td>−0.08 ± 0.60</td>
<td>0.28 ± 0.50</td>
</tr>
</tbody>
</table>

<sup>a</sup> % of antigen-specific RFC = % of test antigen RFC − % of human serum albumin RFC for background correction.

<sup>b</sup> KLH, keyhole limpet hemocyanin; ND, not done.

<sup>c</sup> Keyhole limpet hemocyanin-specific antibody extracted from keyhole limpet hemocyanin-immune donors by affinity chromatography.

<sup>d</sup> Donor mononuclear cells were cultured overnight at 37°C (RPMI 1640:5% NCS).

<sup>e</sup> Arming serum is derived from pooled plasma from SQCC-responsive patients.

<sup>f</sup> Mean ± S.D.

<sup>g</sup> Values are statistically significant after background correction (Student’s t test, p < 0.05).

Table 3

Effect of antiimmunoglobulin treatment on tumor-specific rosette formation in SQCC patients

<table>
<thead>
<tr>
<th>Experiment subject</th>
<th>Stage</th>
<th>Treatment</th>
<th>Specific rosette formation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SQCC RFC</th>
<th>Melanoma RFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T&lt;sub&gt;n&lt;/sub&gt;N&lt;sub&gt;m&lt;/sub&gt;</td>
<td>None</td>
<td>0.38 ± 0.12&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.04 ± 0.05</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>T&lt;sub&gt;n&lt;/sub&gt;N&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Anti-Ig&lt;sup&gt;d&lt;/sup&gt;</td>
<td>−0.10 ± 0.22</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>T&lt;sub&gt;n&lt;/sub&gt;N&lt;sub&gt;m&lt;/sub&gt;</td>
<td>None</td>
<td>0.62 ± 0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.09 ± 0.37</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>T&lt;sub&gt;n&lt;/sub&gt;N&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Anti-Ig&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.04 ± 0.14</td>
<td>0.03 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>T&lt;sub&gt;n&lt;/sub&gt;N&lt;sub&gt;m&lt;/sub&gt;</td>
<td>None</td>
<td>0.50 ± 0.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.05 ± 0.31</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>T&lt;sub&gt;n&lt;/sub&gt;N&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Anti-Ig&lt;sup&gt;d&lt;/sup&gt;</td>
<td>−0.14 ± 0.15</td>
<td>0 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>T&lt;sub&gt;n&lt;/sub&gt;N&lt;sub&gt;m&lt;/sub&gt;</td>
<td>None</td>
<td>1.06 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.03 ± 0.21</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>T&lt;sub&gt;n&lt;/sub&gt;N&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Anti-Ig&lt;sup&gt;d&lt;/sup&gt;</td>
<td>−0.13 ± 0.08</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Specific rosette formation = % of tumor antigen RFC − % of human serum albumin RFC.

<sup>b</sup> Mean ± S.D.

<sup>c</sup> Statistically significant response after background correction (Student’s t test, p < 0.05).

<sup>d</sup> Goat F(ab’)_2 with anti-human immunoglobulin (polyvalent) specificity; ND, not done.

DISCUSSION

We studied the tumor-specific recognition responses of SQCC patients by quantitation of antigen-dependent RFC. The flow cytometeric technique, which uses multiple replicas and large sampling sizes to establish the low frequencies of RFC, has been a considerable technical improvement over the conventional microscopic techniques. Furthermore, responses of several patients to a tumor antigen panel could be examined on the same day. In this study, tumor-specific responses were detected in over >70% of the SQCC patient population with primary cancer, but no activity was detected.

pared the arming capacity of pooled antisera from responders and patients with recurrent tumors on normal cells (Chart 6). Control cells acquired an SQCC RFC response after preincubation with either whole sera (data not shown) or 50 μg (0.27 ± 0.02%) and 500 μg (0.49 ± 0.20%) of pooled sera IgG (DEAE-Fraction I) from responsive patients per 4 × 10<sup>6</sup> cells per ml (Chart 6A). Preincubation with 5 μg/4 × 10<sup>6</sup> cells/ml was ineffective. Specificity of the arming response was suggested by the lack of reactivity to melanoma after incubation with the enriched IgG. Despite the large response variances after preincubation with 500 μg Fraction I (which may be attributable to the small sampling size), the acquired response was significant (p < 0.05). The IgG-depleted fraction (Fraction II) did not have an arming effect within the range of concentrations (5 to 500 μg) tested.

In contrast, neither the pooled sera from patients with recurrent tumor nor either of the constituents from the DEAE-fractionation (IgG-enriched Fraction I and IgG-depleted Fraction II) demonstrated any arming ability on normal cells (Chart 6B).
among patients with recurrent tumor. Since reactivity of this assay is related directly to immune recognition, the routine monitoring of RFC reactivity could be informative for therapeutic or prognostic considerations.

Specificity of the tumor antigen rosette-forming response to the SQCC antigen extract was implied by the low frequency of cross-reactivity in the control groups to the SQCC tumor extract and SQCC-responder patients to other tumor extracts. Thus, it is unlikely that rosette formation was directed towards common contaminating antigens, such as B2 microglobulin, HL-A, or carcinoembryonic antigen (4, 13, 14), as these components are present in both SQCC and the control tumor extracts. In addition, rosette formation to the relevant extract could be inhibited by preincubating responder cells with SQCC but not other tumor antigens. Our preliminary study of a melanoma patient study group also demonstrated a high frequency of melanoma-specific rosette-forming response lacking cross-reactivity to other tumor extracts, including SQCC (data not shown). These observations are in agreement with other studies (1–3, 5, 9, 22, 24, 25) that demonstrated tumor-specific responses in vitro by using 3 M KCl tumor extracts.

The effect of therapeutic regimens, including chemotherapy and irradiation, has not been a critical concern in the present study. Almost all patients with a newly diagnosed primary cancer were evaluated prior to medication or treatment. Patients with recurrent tumor were also evaluated before treatment, although we cannot exclude possible long-term effects from prior treatments. The majority of patients in remission were tested while under maintenance medication, but this apparently did not diminish their rosette-forming response.

Experiments characterizing tumor-specific RFC suggested that they are primarily cytophilic IgG-bearing monocytes. Although at least 80% of monocytes carry Fc receptors for IgG (17, 21), the low frequency of detectable RFC suggests that we may be selectively detecting monocytes bearing Fc receptors of high avidity for IgG against tumor antigens (21), considering the low-epitope density of tumor-specific antigens in the 3 M KCl extracts. Attempts to enhance the recovery of rosettes by glutaraldehyde fixation (data not shown) or the routine enrichment of RFC by isopyknic centrifugation have not been successful, possibly due to the inability of rosettes to withstand the rigors of washing procedures. We are examining presently alternate inert carriers for tumor antigens, as well as using other tumor antigen extraction procedures to enhance the detection of RFC. Further experiments are also required to identify the predominant IgG subclass of the mediating antibodies and the Fc specificity of the participating monocytes.

Various studies have indicated that the antibody-bearing monocytes are functionally heterogenous and may be comprised of subpopulations responsible for antigen presentation (11, 17, 19), immunoregulation (11, 12), and tumor cytotoxicity and rejection responses (17, 18, 21, 26, 28). The possible correlation between monocyte function and antibody avidity, however, has not been defined clearly (11, 21). Although we have yet to establish the functional capability of the tumor-specific RFC, the apparent correlation between tumor recurrence and loss of tumor-specific RFC reactivity is interesting. Conversely, other studies (16, 20) have demonstrated improved survival of tumor-laden mice following the administration of hyperimmune tumor-specific antibody. We favor the hypothesis that these RFC may represent a functionally distinct monocyte subpopulation that is involved in tumor recognition and/or rejection processes.

In a longitudinal study, tumor-specific RFC were detected in a patient before and after primary tumor resection. However, RFC reactivity was not detected when he was presented with tumor relapse at 6 months (data not shown). This patient, and other patients with tumor recurrence, had normal or elevated monocyte counts (15 ± 3%) but remained anergic even after efforts to free complexed receptors through cell culturing in vitro. In a study that compared antigen binding between high- and low-responder mouse strains, low responders were characterized by the absence of a monocyte subpopulation that can acquire cytolytic antibodies for antigen-specific recognition (10). A similar defect may exist in anergic patients. However, the alternative of depressed monocyte function by tumorsecreted materials other than immune complexes cannot be excluded (21).

Several points in this study are yet unclear, including (a) if the level of RFC reflected the strength of a tumor-specific immune response of a patient and, more importantly, (b) whether nonresponders among the primary cancer population have a higher chance of tumor recurrence than do responders. The latter point is being investigated in ongoing longitudinal studies. These studies may help establish the in vivo relevance of tumor-specific RFC.

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Flow Cytofluorometric Detection of Tumor-specific Rosette-forming Cells in Patients with Squamous Cell Carcinoma of the Head and Neck

Alex W. Tong, Arthur A. Vandenbark, William Kraybill, et al.


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