Humoral Immune Response of Patients Receiving Specific Active Immunotherapy for Renal Cell Carcinoma

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ABSTRACT

The object of this study was to characterize the antigens evoking an immune response in renal cell carcinoma (RCC) patients receiving specific active immunotherapy with irradiated autologous tumor cells and Corynebacterium parvum as adjuvant. Seventy serum samples from 11 patients with RCC undergoing specific active immunotherapy were evaluated. Fifty of the 70 serum specimens (71%) had immunoglobulin G anti-antigen present on their autologous tumor cells. One patient's serum (R. N.) was absorbed not only with autologous tumor cells but also with an allogeneic RCC cell line. The fourth patient's (S. E.) serum reactivity was able to be absorbed only with autologous tumor cells and several, but not all, of the clones of that autologous cell line. Patient S. E. serum binding by clones of RCC cell line RPMI-SE was seen to vary from no ability to bind RPMI-SE in some clones to double the parental binding in others. Consistent with this finding was the demonstration that high serum-binding clones could absorb Patient S. E. serum reactivity to autologous RCC cells, while low binding clones could not. These data suggest a measure of heterogeneity among the antigens present on these autologous tumor cells. The identification of antigens evoking an immune response in patients receiving SAI for RCC. A MHA using autologous tumor vaccine with adjuvant has shown that the autologous tumor vaccine with adjuvant used here was an immunogenic therapeutic agent. The response mounted by these patients was a response to a RCC-associated antigen with the level of reactivity changing with the number of immunizations and disease status. Also suggested by this work is the possible primary tumor heterogeneity, as demonstrated by the differential reactivity seen among clones of a RCC cell line established from such a primary tumor.

INTRODUCTION

The identification of antigens evoking an immune response during the course of SAI for malignant disease is important in the assessment of the effectiveness of the treatment. Approximately 2000 patients have been treated with this form of therapy, both with and without adjuvants, in the past decade (1). Several questions concerning serological specificity, as opposed to reactivity to histocompatibility antigens, are answered by the use of autologous typing procedures developed by Old (2) and co-workers. This method of assessing serological specificity makes exclusive use of autologous tissue as the source of antigen, thereby avoiding detection of antigens normally present on cells and subject to misleading cross-reactivity in allogeneic systems. A classification system for tumor antigens, based on serological specificity, has been developed (2). This system provides a method of determining the type of antigen(s) being responded to, and the extent to which the antigen(s) cross-react. Briefly, Class I antigens are unique and restricted to the autologous tumor; Class II antigens share specificity with a restricted number of similar tumors, with some antigens cross-reacting with differentiation antigens and tumors of other types; finally, Class III antigens are cross-reactive with a wide variety of antigens usually associated with passive acquisition subsequent to the growth of cells in FCS. Four systems have been studied by this methodology: melanoma (3-8); astrocytoma (9); RCC (10), and leukemia (11). To date, 7 Class I and 13 Class II antigens have been demonstrated from 130 patients, with the remainder being Class III. The present study concerns the nature of the immune response in patients receiving SAI for RCC. A MHA using autologous tumor targets demonstrated a tumor-associated antigen present on RCC cells of most of the patients tested. Classification of the antigens playing a role in those responses and evidence of primary tumor heterogeneity is presented.

MATERIALS AND METHODS

Sample Collection and Storage. Serum was collected from whole blood samples drawn from patients throughout their course of immunotherapy and at follow-up visits thereafter. Serum samples were aliquoted and frozen at −70°C until used. All samples were centrifuged (200 × g for 10 min) free of cellular elements prior to use.

MHA. The MHA assay used throughout these experiments was the method of Espmark and Fagerus (12), as modified by Liao et al. (13). Briefly, autologous RCC cells (2 × 10^6/ml) were plated into 60-well microtiter plates (NUNC, Denmark), using a 1-cc tuberculin syringe fitted with a 26-gauge needle and incubated overnight at 37°C in a humid 5% CO2 in air atmosphere. This yielded a cell concentration of 100 to 200 adherent cells/well. The plates were washed gently with phosphate-buffered saline and 5% FCS ( Gibco). Serial-doubling dilutions of the test serum were plated in replicates of 6 and incubated at room temperature for 2 h. The indicator system consisted of human type A erythrocytes incubated with a subagglutinating concentration of human anti-human blood group antigens A and B (Ortho Diagnostic Systems, Inc., Ranttan, NJ), followed by washing and incubation with affinity-purified goat anti-human IgG (γ chain specific; Antibodies, Inc., Davis, CA) at a subagglutinating concentration. The completed indicator system was added to the test plate, which had been washed free of excess test response to these antigens have not withstood close scrutiny (1). Several questions concerning serological specificity, as opposed to reactivity to histocompatibility antigens, are answered by the use of autologous typing procedures developed by Old (2) and co-workers. This method of assessing serological specificity makes exclusive use of autologous tissue as the source of antigen, thereby avoiding detection of antigens normally present on cells and subject to misleading cross-reactivity in allogeneic systems. A classification system for tumor antigens, based on serological specificity, has been developed (2). This system provides a method of determining the type of antigen(s) being responded to, and the extent to which the antigen(s) cross-react. Briefly, Class I antigens are unique and restricted to the autologous tumor; Class II antigens share specificity with a restricted number of similar tumors, with some antigens cross-reacting with differentiation antigens and tumors of other types; finally, Class III antigens are cross-reactive with a wide variety of antigens usually associated with passive acquisition subsequent to the growth of cells in FCS. Four systems have been studied by this methodology: melanoma (3-8); astrocytoma (9); RCC (10), and leukemia (11). To date, 7 Class I and 13 Class II antigens have been demonstrated from 130 patients, with the remainder being Class III. The present study concerns the nature of the immune response in patients receiving SAI for RCC. A MHA using autologous tumor targets demonstrated a tumor-associated antigen present on RCC cells of most of the patients tested. Classification of the antigens playing a role in those responses and evidence of primary tumor heterogeneity is presented.
serum, for 1 h at room temperature. The test plates were gently washed and fixed with 0.25% glutaraldehyde to preserve rosettes. Scoring was accomplished via a Visopan projection microscope (Reichert, Vienna, Austria) visualizing entire wells. Cells having 5 or more indicator cells attached were scored as positively rosetted.

Primary Culture and Establishment of RCC Cell Lines. Autologous target cell lines were established by a sequential enzymatic disaggregation procedure. Briefly, surgical specimens grossly freed of necrotic and normal tissue prior to acquisition for immunotherapy preparation were minced and stored in RPMI 1640 (GIBCO), 20% FCS, and 10% dimethyl sulfoxide (Eastman Kodak, Rochester, NY) in liquid nitrogen until used. Tumor chunks were quick thawed and washed extensively with RPMI 1640 and 20% FCS to remove dimethyl sulfoxide, followed by further washings with serum-free RPMI 1640 to remove FCS. Tumor chunks were then placed into a dissection flask containing collagenase (250 units/ml; Worthington Biochemical Corp., Freehold, NJ), and hyaluronidase (0.12 g; Sigma Chemical Co., St. Louis, MO) at 37°C in a shaking water bath for 45 min. The enzyme mixture was removed and Pronase (1%; Calbiochem, La Jolla, CA) was added to the flask for an additional 45 min at 37°C with agitation. DNase (Worthington) was then added and the tumor cell suspension was filtered through glass wool to remove large debris. Cells were diluted with RPMI 1640, 10% FCS, and 0.1 unit insulin/ml (E. R. Squibb & Sons, Princeton, NJ), overlaid onto Hypaque-Ficoll (Nyegaard, Norway), and centrifuged at 200 x g for 20 min. The interface was recovered, washed with culture media, and the cells were placed into 25 cm² culture flasks. Growth was monitored closely with media changes at 24 h, and as culture conditions required thereafter.

Limiting dilution Cloning. Cloning of RPMI-SE was accomplished by limiting dilution. The cell suspension to be cloned was adjusted to 10 cells/ml, and 0.1 ml was placed into each well of 96-well culture plates (COSTAR, Cambridge, MA). Three to 4 h postseeding each well was assessed by inverted microscope at ×100 for the presence of cells. Those wells containing only one cell were noted for further study.

Serum Absorptions. All absorptions were carried out using patient serum collected on dates of peak serum reactivity, as determined by sequential MHA studies. Qualitative absorptions were accomplished by harvesting sufficient quantities of cultured cells to create a dry cell pellet of volume approximately equal to that of the test serum to be absorbed. Cells were harvested by trypsinization (Trypsin-EDTA; GIBCO) and adjusted to 2 x 10⁶/ml. Membrane reconstitution was accomplished by 2 consecutive 3-h incubations of cells in tissue culture medium supplemented with 20% FCS. Cells were placed in a sterile, siliconized flask, and were incubated in a 37°C water bath with slow agitation. Serum to be absorbed was diluted with RPMI 1640 (GIBCO) 2 doubling dilutions below the peak rosette-forming dilution, admixed with the absorbing cells, and incubated overnight at 4°C.

Quantitative absorptions were completed by adjusting absorbing cell concentrations to 5 x 10⁵, 1 x 10⁶, 5 x 10⁶, 1 x 10⁷, 7.5 x 10⁷, 5 x 10⁸, 2.5 x 10⁹, and 1 x 10¹⁰ cells/absorption. Two ml of test serum previously diluted as described above were divided equally among the absorbant cell concentrations and incubated as above.

Corynebacterium parvum (COPARVAX, Burroughs Wellcome Co., Research Triangle Park, NC) absorptions were completed in a qualitative fashion by pelleting 8.4 mg of formalin-killed vaccine preparation at 15,000 x g for 4 min. Diluted test serum was used on a 1:1 (v/v) basis to resuspend the C. parvum pellet, and absorptions were carried out as above.

Normal buffy coats were obtained from the RPMI Blood Bank from plasmapheresis volunteers. All donors had been HLA phenotyped previously. For absorptions a variety of phenotypes were chosen, mixed, and used as qualitative absorptive material.

Absorbed serum was tested for binding in an autologous MHA, using a dilution of serum equal to that which yielded maximum binding of nonabsorbed serum. Nonabsorbed serum was always included at the same dilutions as a positive control.

Patient Selection. The patients studied herein were all participants in a randomized clinical protocol examining the effect of SAI on RCC. The immunotherapy consisted of s.c. injections of C. parvum (35 µg/10⁷ cells) plus irradiated (15,000 rads) autologous tumor prepared as a single-cell suspension by the identical method used to establish autologous RCC cell lines described above, administered approximately every 14 days. No cultured cells were used at any time as a source of autologous tumor cells. All patients were assessed as Stage IV at the time of study and all had undergone a nephrectomy. Selection for the present study was based solely on the ability to grow autologous tumor for use in the MHA assay.

RESULTS

Growth and Morphology of RCC Cell Lines. All but one specimen originated from a primary tumor, the remaining specimens being taken from a metastatic growth. The histology of the specimens was largely of a clear cell type, with the few remaining specimens termed mixed clear-cell and granular tumors. Seventy-one % of the specimens received exhibited primary, nonfibroblastic growth. Primary growth was defined as the ability of a culture to be passaged at least once. Seventeen % of the attempts resulted in fibroblastic growth which was not suitable for use in the MHA assay. Eleven % of the attempts were completely unsuccessful; the single specimen of metastatic origin was in this unsuccessful category. Approximately 50% of the primary cultures could be passaged 3 times. Fifteen % of the cultures were successfully maintained beyond the sixth passage, and continued unabated beyond that point.

The morphology of cells in culture fell into one of 3 categories, as described by Ueda et al. (10), with each cell line demonstrating only one type. Of the cultured lines, 52% demonstrated a pattern of compact, epithelial cells with irregular, sharply defined edges. Of the cultured lines, 28% demonstrated a pattern of cell growth containing globular-shaped cells with pseudopod-like structures. Twenty % of the cell lines demonstrated spindle-shaped cells, usually found growing in layers.

Survey of Serum Binding to Autologous Tumor Cells. An autologous typing survey was completed in order to study the antibody response of patients undergoing SAI for RCC. Serum samples taken on 70 different occasions from 11 patients receiving SAI were tested against autologous RCC cells using the MHA assay. Of the 70 serum dates tested, 50, or 71.4%, demonstrated binding to autologous RCC cells. Of the 11 patients receiving SAI, only 2 failed to show some autologous reactivity. Of the 9 patients demonstrating reactivity to autologous RCC cells, 4 were found to have sufficient numbers of serum samples over time and to have suitable numbers of cells to support a more detailed study of their reactivity. None of these 4 patients demonstrated serum reactivity to autologous RCC cells in the interim following nephrectomy and prior to SAI (data not shown).

Autologous and Allogeneic Absorption of Serum Samples. Serum samples from 4 patients were absorbed with a number of autologous and allogeneic cell types, both malignant and normal. Although the use of an autologous test system virtually precludes reactivity across histocompatibility barriers, the possibility of serum-binding activity in this area was also investigated. Absorptions were also done using the adjuvant C. parvum administered during SAI to determine to what extent, if any, the reactivity toward autologous RCC cells was a function of a response to this strong antigen.
A number of absorptions were completed using the serum of patient S. V. Qualitative absorption of serum with autologous RCC cells produced an 87% inhibition of serum binding. The absorption of serum with the cells, cell lines, and adjuvant listed in Table 1 failed to produce any inhibition of serum reactivity.

Qualitative absorption of patient R. N. serum with autologous RCC cells was able to reduce the serum-binding ability by 70%. Quantitative absorption of R. N. serum by the allogeneic RCC cell line RPMI-MM was also able to inhibit serum binding to a maximum of 78% with $5 \times 10^7$ absorbing cells. No inhibition of R. N. serum binding was demonstrated when other allogeneic RCC cell lines, non-RCC cell lines, normal autologous Buffy coat cells, pooled allogeneic Buffy coat cells, or C. parvum were used as absorbants.

Patient M. M. serum was absorbed with an autologous RCC cell line and a number of allogeneic RCC cell lines, allogeneic non-RCC cell lines, normal autologous Buffy coat cells, and C. parvum. With the exception of the autologous line which completely absorbed serum reactivity, none of these absorbants was seen to produce any inhibition of serum binding to autologous RCC cells.

Chart 1 demonstrates the number of autologous RCC cells needed to absorb Patient S. E. serum. Also depicted in Chart 1 is the result of quantitative absorptions using clones RPMI-SE-2, RPMI-SE-17, and RPMI-SE-27 of the autologous RPMI-SE cell line. These clonal lines were also able to inhibit the serum reactivity of Patient S. E. Absorptions completed using materials listed in Table 1 were unable to remove the rosette-forming ability of Patient S. E. serum in the MHA assay. A total of 6 clones of RPMI-SE were used as absorbants of Patient S. E. serum. Three clones, listed in Chart 1, were able to absorb serum reactivity. The remaining 3 clones, RPMI-SE-1, RPMI-SE-4, and RPMI-SE-10, were unable to absorb reactivity. A more detailed examination of this clonal heterogeneity follows.

Primary Tumor Heterogeneity as Seen in Clones of RPMI-SE. The finding that less than 100% of any cell population was able to form rosettes (peak rosette formation ranged from 45 to 77%; data not shown) in an autologous MHA assay (data not shown) suggested that not all the cells of a given population were expressing a particular antigen at all times. To test this hypothesis, a series of clones were isolated from the RCC cell line RPMI-SE and assessed for serum-binding capacity, as compared to the parental line. Each clone was compared side by side to the parental line in an autologous typing MHA assay experiment, using an aliquot from a single serum date. The results are presented in Chart 2 as the number of clones at a given serum-binding percentage of the parental line. The majority, 15 of 26, are within 40% of the parental line. Three of the clones (RPMI-SE-1, RPMI-SE-4, and RPMI-SE-10) did not demonstrate serum binding at all, while 3 clones (RPMI-SE-2, RPMI-SE-17, and RPMI-SE-27) demonstrated greater than 140% of the serum-binding capacity of the parental line. As was presented in the previous section, clones RPMI-SE-2, RPMI-SE-27, RPMI-SE-10, RPMI-SE-1, RPMI-SE-17, and RPMI-SE-4 were used as autologous absorbants to test the presence of antigen on the surface of the nonbinding clones, RPMI-SE-1, RPMI-SE-4, and RPMI-SE-10, in a more sensitive manner. Only clones RPMI-SE-2, RPMI-SE-17 and RPMI-SE-27 were able to absorb the serum-binding activity of Patient S. E. serum, indicating that antigen is either not present on clones RPMI-SE-1, RPMI-SE-4 or RPMI-SE-10 or is at undetectable levels.

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<tr>
<th>Allogeneic RCC cell lines, allogeneic non-RCC malignant cells, and nonmalignant cells and adjuvant used as absorbants of patient sera</th>
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<tr>
<td>Allogeneic RCC</td>
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<td>RPMI-MM</td>
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<td>A498</td>
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<td>RPMI-RN</td>
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<td>Nonmalignant cells and adjuvant</td>
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<td>Normal autologous Buffy coat</td>
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<td>Pooled normal allogeneal Buffy coat</td>
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<td>C. parvum</td>
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<tr>
<td>Allogeneic non-RCC malignant cells</td>
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<td>LN 229 (Astrocytoma)</td>
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<td>PaCa-2 (pancreatic carcinoma)</td>
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<td>RPMI 7451 (pancreatic carcinoma)</td>
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Chart 1. Absorption of Patient S. E. serum with autologous RCC cell line RPMI-SE and clones of RPMI-SE. O, RPMI-SE-2; C, RPMI-SE-17; A, RPMI-SE-27; S, RPMI-SE.
**DISCUSSION**

In order to judge sufficiently the effect of any mode of therapy, it is necessary not only to monitor the patient’s progress on a clinical basis, but also to judge the response of the patient independently. The objective assessment of therapeutic response to immunotherapy directed against malignancy has remained a difficult task. Immunological assays measuring nonspecific parameters of therapeutic response or immunocompetence are, by nature, unable to answer all questions about effectiveness of a particular mode of therapy in relation to an individual’s tumor. It is, therefore, essential to the critical evaluation of any immunotherapeutic protocol to assess a given patient’s response to therapy in the context of how, or if, the autologous tumor is being responded to immunologically. The establishment of immunotherapeutic agents as immunogenic has remained elusive, with regard to SAI, as the definition of immunogenicity of the vaccine has been hampered by the inability to separate a specific immune response from the response to stronger nonspecific antigens. This difficulty in detecting specific immune response led Livingston et al. (1) to conclude that a specific tumor-associated antigenic response as a result of SAI is a rare event.

That the vaccine used in the present work was immunogenic in most patients was clearly demonstrated in 71.4% of the serum collections. The serum reactivity to autologous cells seen among the patient population studied offered some contrasts to a similar study by Ueda et al. (10). The survey that they completed failed to identify more than one positive reaction out of 22 patients reacted with autologous typing and the MHA assay. Other hemadsorption assays used were able, in high proportion, to identify patients giving positive reactions to autologous cells. The discrepancy from Ueda’s work may be explained by the fact that all patients included in this work were receiving SAI at the time of collection. This would have resulted in increased immune stimulation, presumably increasing the specific IgG content in the serum, resulting in a greater proportion of positive responses. With regard to the other assays used in Ueda’s work, the responses detected by these assays are, in 2 of the assays, IgM responses not detectable by the MHA assay. The key point made by both Ueda’s survey and the present study remains that RCC patients do respond in a specific manner to autologous RCC cells.

The 4 patients selected for further study were representative of the patient population as a whole. One patient (S. E.) remains a complete responder to therapy, while another (R. N.) was a complete responder and later progressed. Patient M. M. was assessed as no better than clinically stable throughout the therapeutic course, and the final patient (S. V.) never clinically responded to immunotherapy. This group of patients effectively covered all realms of clinical responses to the protocol. Thus, it may be expected that their serum responses may reflect that general patient population as well.

Absorption studies are the most sensitive technique available for testing the specificity of the response to the immunogenic vaccines. Several studies have, upon extensive absorption, failed to identify a specific response (1, 14, 15) or have identified such specific antigens only rarely (3, 10, 16). Studies involving SAI using cultured cells, either autologous or allogeneic, have been hampered by the expression of antigens related to the growth of cells in tissue culture medium containing FCS (1, 14). The present work avoids sensitizing the patients to such antigens by using only fresh frozen, extensively washed, tumor chunks for SAI. The absorption studies that were completed served a 2-fold purpose. Absorptions of reactive serum by a battery of cell types would answer the question of SAI specificity. In addition, absorptions of this type could attempt to classify the antigen(s) being responded to.

A tentative antigenic classification may be made on the basis of the absorption data. Serum collected from Patient S. V. appears to be recognizing a Class I antigen because only the autologous RCC cell line RPMI-SV, and no other cell types, were able to absorb any activity. A Class I antigen is likewise apparently being recognized by serum from Patient M. M., as all attempts at absorbing serum-binding activity were unsuccessful, with the single exception of the autologous RCC cell line RPMI-MM, which could absorb activity. Firm classification of Class I antigenicity is not warranted at this time, however, due to the relatively limited absorption capability of this study. This work has, nonetheless, documented a serological response to a tumor-associated antigen whose fine specificity remains undetermined. In contrast, serum from Patient R. N. appears to have recognized a Class II antigen. In addition to the autologous absorption, cells from the allogeneic RCC cell line RPMI-MM were able to significantly absorb serum-binding activity directed against the autologous RCC cell line RPMI-RN. All other allogeneic RCC cell lines and other malignant and normal cells used as absorbants were unsuccessful in absorbing reactivity, thus classifying it by definition as a Class II antigen. Antigenic classification based on the serum reactivity of Patient S. E. is more complex. While binding ability directed against the autologous RCC cell line RPMI-SE was able to be absorbed with that autologous cell line and no allogeneic cell line, it was not the only cell line capable of absorption. Three clones of RPMI-SE, RPMI-SE-2, RPMI-SE-17, and RPMI-SE-27 were equally capable of absorbing the binding ability of serum from Patient S. E. Therefore, it appears that some of the clones express the parental antigen while others do not, or express it in nondetectable quantities. Cell cycle dependency of antigenic expression would...
result in such expression in a proportion of the cultured cells at all times. As all clonal cultures used were randomly growing populations, and some of the clones did not demonstrate serum binding at all, cell cycle-dependent expression of antigen as the basis of the heterogeneity seen was not supported. Thus, the definition of a Class I antigen as an antigen detectable on only the autologous tumor cells might be amended, or expanded, to include certain clones of the parental line also. Absorptions using normal autologous kidney tissue were not able to be completed due to unavailability of normal tissue to the investigators. However, while reactivity to normal kidney tissue antigens cannot be formally excluded, the possibility is deemed to be limited for the following reasons. Data concerning serum reactivity following nephrectomy demonstrated a decrease in serum binding prior to administration of SAI. Serum reactivity was seen to increase with vaccine administration in all patients. As the vaccine is composed of autologous tumor tissue following sequential enzymatic disaggregation, it is considered unlikely that an increase in serum-binding ability to autologous cultured malignant cells could be the result of normal tissue antigen sensitization. In order for a response to have been made to a normal autologous tissue antigen, immunological unresponsiveness to such antigens on the contralateral kidney would have to have been overcome. In support of this is the lack of clinical repercussions such as glomerulonephritis or autoimmune complications, which may have been expected if vaccine administration had resulted in sensitization to a normal kidney tissue antigen presumably present on the contralateral kidney.

The isolation of clones of RPMI-SE and their variable reactive and absorptive capabilities supports the concept of heterogeneity among primary tumors, first hypothesized by Henderson and Rous (17). The variability among clones with respect to serum reactivity and absorptive capability may be part of the reason that metastatic growth persists after a period of time in most patients receiving SAI. It is possible that the antibody produced as a result of immunotherapy does have an effect on a portion of the metastatic cells which express the parental antigen. It is equally possible, therefore, that metastatic cells not expressing the parental antigen may escape the effects of immunotherapy completely, only to grow out at some future date, leading to progressive disease. Rogers (18), in a review of TATA, proposed that while tumors may express multiple TATA, the total possible number of TATA is probably not large. This is supported by Chart 2, in that over one-half of all isolated clones are within 40% of the parental line binding. This demonstrates that a low number of isolated clones are completely at variance with the original culture. Also suggested by Rogers was the possibility of a regulatory mechanism for the response to some TATA in the presence of strong TATA. If this is in fact the case, then SAI as a treatment modality is in question, as the active specificity it brings to the patient may prevent the immune system from recognizing and possibly neutralizing the entire tumor.

Clones isolated from RCC cell line RPMI-SE demonstrated the heterogeneity of the parental line through their variability in serum reactivity and absorbant ability. This may provide an explanation for the high frequency of disease progression seen over the length of the protocol. Immunotherapy design must, therefore, include a more complete understanding of how the entire immune system recognizes and responds to tumor-associated antigens, and why this response fails to halt tumor progression initially. Further work is also needed concerning the identification of tumor-associated antigens, and the study of the biology of tumor growth with respect to defense mechanisms.

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