T-Lymphocytes and Macrophages in Primary Murine Fibrosarcomas at Different Stages in Their Progression

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ABSTRACT

The relative contribution of lymphocytes, macrophages, and granulocytes to the cell content of primary 3-methylcholanthrene-induced murine fibrosarcomas was determined at different stages in their progression by differential cell analysis on enzyme-derived single-cell suspensions. Furthermore, immunohistological analyses were performed on the tumors to detect, quantitate, and determine the distribution of T-lymphocytes and macrophages. The T-lymphocyte content of small tumors was very high, and the T-cells were distributed throughout the tumor mass. As the tumor increased in size, there was a marked decrease in the relative T-cell content; most were located at the tumor periphery. Macrophages were present in significant numbers in all tumors and appeared to increase in number as the tumors increased in size. Macrophages were distributed throughout the tumor mass, but generally they were more densely distributed near the tumor periphery. Granulocytes were present in low numbers in all tumors. Yeast phagocytosis was used to assess the functional capacity of the macrophage population. The phagocytic capacity of the macrophages was low in the small tumors, increased significantly as the tumors progressed, but dropped to relatively low levels in large tumors. The results represent a preliminary attempt to characterize the dynamics of host cell infiltration of primary immunogenic tumors.

INTRODUCTION

The cellular composition of solid nonlymphoreticular neoplasms until recently has been assumed to be primarily malignant cells. Such an assumption was made despite the fact that the malignant cells were known to possess antigenic determinants to which the host responded immunologically (5, 17). The reason for such a supposition was that the immune response exerted no apparent effect on the primary tumor, and the tumor grows progressively even though immunity may be demonstrated concomitantly (22) subsequent to tumor excision (5, 17) or following immunization with killed tumor cells (19). Nevertheless, several studies have demonstrated that both chemically and virally induced experimental tumors (3, 8, 11, 26) and spontaneous human tumors (6, 28) contain large numbers of lymphoreticular cells (primarily macrophages) which may represent a tumor-associated manifestation of the host anti-tumor cellular immune response. Although the presence of significant numbers of lymphoreticular cells within solid tumors could represent a nonspecific inflammatory response, the fact that the size of the tumor-associated macrophage population has been shown to be directly related to tumor immunogenicity suggests that the host cells are attracted by immunological mechanisms (2). Furthermore, T-lymphocytes derived from tumors have been shown to have specific antitumor activity (7, 10, 16), and macrophages derived from tumors were shown to be either tumorstatic (4, 8, 23) or tumoricidal (9, 21). Those studies provide preliminary circumstantial evidence for the existence of a tumor-associated immune response.

Previous studies have concentrated on passaged tumors and have not systematically evaluated changes in the infiltrating host cells as the tumor progresses. Furthermore, enzymatically derived tumor cell suspensions have been used, and there have been no studies that correlated those data with the relative proportion of host cells in the intact tumor. Thus, the present study was designed to examine a large number of primary tumors at different times following their initial appearance both through cellular analysis of enzymatically derived tumor suspensions and through immunohistological analysis of the intact tumor with specific cell antisera.

MATERIALS AND METHODS

Tumors

Primary tumors were induced s.c. in 5- to 6-week-old male C3H/He mice by injection of 0.5 mg of MCA dissolved in 0.1 ml of triactanoin. The tumors appeared at the injection site 3 to 12 months later, over 90% appearing within 3 to 5 months. Tumors that are induced s.c. with MCA almost invariably remain localized but progress steadily to kill the host. Because the study was designed to evaluate cell composition at different points in tumor development, the tumors were allowed to progress for varying periods of time between their appearance and surgical removal. Thus a continuum of tumor sizes was achieved from those that were very small at the time of removal (<0.25 cu cm) to those that were relatively large (>1.0 cu cm). The size of the intact tumor was determined from 3 measurements of tumor diameter with the formula 0.52 cde, which is the formula for the volume of an ovoid object.

When the tumor was excised care was taken to ensure that most if not all malignant tissue was removed; then the tumor was divided. A portion was prepared for enzymatic digestion as described below and the remainder was snap frozen in liquid N2 and stored in sealed vials at −70°.

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Antisera

**Rabbit Anti-Sheep RBC Serum.** The rabbit anti-sheep RBC (Baltimore Biological Laboratories, Cockeysville, Md.) had an agglutination titer of 8000 and was composed primarily of IgG-type sheep RBC antibodies as determined by passive agglutination with goat anti-rabbit γ-globulin.

**Goat Anti-Rabbit γ-Globulin.** Goat anti-rabbit γ-globulin (Miles Laboratories, Kankakee, Ill.) was conjugated with FITC as described previously (25). The antiserum failed to cross-react with mouse immunoglobulin in Ouchterlony gel diffusion analyses.

**Rabbit Anti-Mouse T-Lymphocyte Serum.** Rabbits were immunized with CBA mouse brain emulsified in Freund’s adjuvant. The antiserum was absorbed v/v with C3H liver homogenate and v/v with BALB/c plasmacytoma homogenate (MOPC104E and MOPC195). The absorbed antiserum killed >95% C3H thymocytes and 30 to 50% of the cells in suspensions of unfractionated C3H splenocytes. Furthermore, in indirect immunofluorescence assays with the antiserum, >95% C3H thymocytes, 40 to 50% splenocytes, 50 to 60% lymph node cells, and 10 to 15% peritoneal exudate cells were positively stained. The antiserum had no reactivity for tumor cells, B-lymphocytes, macrophages, or granulocytes. The antiserum failed to react with mouse serum in Ouchterlony gel diffusion analysis. The specificity of the antiserum for T-cells was confirmed independently (Dr. P. Lill, Frederick Cancer Research Center, Frederick, Md.). Additional specificity studies are included under “Results.”

**Rabbit Anti-Mouse Macrophage Serum.** C3H peritoneal exudate cells (thioglycollate-induced) were purified by adherence to plastic Petri dishes. The adherent cells were removed by scraping, emulsified in Freund’s adjuvant, and used to immunize rabbits. The resultant serum was absorbed twice v/v with C3H kidney homogenate. The antiserum was used routinely at a dilution of 1:50 and at that concentration demonstrated minimal reactivity with C3H thymocytes in cytotoxicity (<5%) and immunofluorescence (<10%) assays, produced positive fluorescence with 75% of C3H peritoneal exudate cells, and failed to stain cultured MCA tumor cells. Furthermore, an aliquot of the antiserum was exhaustively absorbed with murine thymus homogenate and tested on several selected tumors. The staining patterns were unaltered. The antiserum failed to react with mouse serum in Ouchterlony gel diffusion analysis.

**Cellular Analysis of Tumor Suspensions**

**Enzymatic Digestion.** Necrotic tissue was removed carefully from the tumor; the tumor tissue was minced, washed twice with Hanks’ balanced salt solution, and suspended in 0.25% trypsin diluted in PBS. Trypsinization was allowed to proceed for 90 min at 22–24° while tissue was stirred in a trypsinization flask (Belco Glass Co., Vineland, N. J.); the cell suspension was freed of undigested fragments by filtration through sterile gauze pads (4 x 4 ply), mixed with an equal volume of Nutrient Mixture F12 supplemented with antibiotics and 20% fetal bovine serum, sedimented at 500 x g for 10 min, and resuspended in the supplemented medium. The cell suspensions that were obtained in this manner consistently had greater than 80% viability as determined by trypan blue exclusion.

**Quantitation of Macrophages.** The percentage of cells in each tumor that were characteristic as macrophages was approximated by enumeration of medium to large Fc receptor-positive cells by a modified EA rosette technique. Previous investigations have established that most if not all Fc receptor-positive cells within tumors were macrophages (18, 26, 28). Sheep erythrocytes were coated with 1 agglutinating unit of rabbit anti-sheep RBC and an appropriate concentration of goat anti-rabbit γ-globulin. Equal volumes of a 0.5% suspension of EA and the tumor cell suspension (1.0 x 10⁶ cells/ml) were mixed, incubated at 37° for 5 min, sedimented at 300 x g for 5 min, and resuspended, and the percentage of medium to large rosetted cells was determined. At least 200 cells were counted, and any cell with at least 3 attached erythrocytes was counted as positive. Routinely, the rosetted cells were cytocentrifuged (Shandon Southern Instruments, Inc., Sewickley, Pa.), stained with Wright’s agent, and examined to verify that most rosetted cells were macrophages.

**Quantitation of Granulocytes and Lymphocytes.** The percentage of the total tumor cell population that was composed of granulocytes or lymphocytes was determined by differential count on cytocentrifuged, stained cell preparations. At least 400 cells were counted for each determination. The percentage of the lymphocyte population that was composed of T-lymphocytes was determined with some of the tumors by comparing the lymphocyte percentage from morphological analysis with the percentage of cells that exhibited positive fluorescence in an indirect immunofluorescent assay with heterologous anti-mouse T-cell serum. The method is described below.

**Assay for Phagocytosis**

A suspension of Saccharomyces sp. was heat-killed (100° for 30 min) and washed twice with PBS. The yeast were enumerated and mixed with tumor cells in a ratio of 10:1. The mixture was incubated at 37° for 60 min, thoroughly mixed, cytocentrifuged, and stained with Wright’s agent. The number of cells that had ingested killed yeast was determined by examination of at least 400 cells. Certain cell types such as lymphocytes and tumor cells lacked the potential for phagocytosis, and it was important to obtain phagocytosis data only on cells that were potentially capable of ingesting the yeast, the granulocytes and the macrophages. Therefore, the phagocytic percentage was determined with the following formula:

\[
\text{Phagocytic percentage} = \frac{\% \text{ phagocytic cells}}{\% \text{ granulocytes} + \% \text{ macrophages}}
\]

**Indirect Immunofluorescent Assays**

**Cell Suspensions.** Anti-T-lymphocyte serum was diluted 1:20 in PBS and incubated at 4° for 20 min with 0.5 x 10⁶ tumor-associated cells; the cells were washed twice with ice-cold Hanks’ balanced salt solution, incubated at 4° for 20 min with FITC-conjugated goat anti-rabbit γ-globulin diluted 1:50, and washed twice with iced PBS. All prepara-
tions were read for membrane immunofluorescence immediately.

**Staining Tumor Tissue Sections.** Sections were cut 4 to 6 μm thick from frozen tissue, placed on slides, and stored for up to 1 week at −20°C. Tissue sections were washed for 30 min with PBS and covered with appropriately diluted specific antiserum (normal rabbit serum, 1:10; anti-T-cell serum, 1:10; and antimacrophage serum, 1:50). The slides were incubated in a moist chamber for 30 min at ambient temperature and then washed twice for 20 min with PBS. The FITC-goat anti-rabbit globulin, diluted 1:50 in PBS, was added to the sections which were incubated in the moist chamber for 30 min and washed twice for 20 min with PBS. The coverslips were then mounted with 90% glycerol:10% PBS.

Immunofluorescence was observed and photographed with a Zeiss fluorescence microscope equipped with an HBO 200 light source, Schott BG 12 excitation filter, and Schott 65 and 50 barrier filters.

**Detection of IgG Fc Receptor-positive Cells in Tissue Sections**

Frozen sections were cut 8 to 12 μm thick and placed on coverglasses (22 x 40 mm). The sections were stored in sealed containers at −20°C and assayed within 1 week.

A 5.0% suspension of sheep RBC was prepared following washing 3 times with Veronal:gelatin buffer, and the suspension was mixed 1:1 with appropriately diluted rabbit anti-sheep RBC, incubated for 30 min at 37°C, washed 3 times with Veronal:gelatin buffer, and diluted to 1.0% in Hanks' balanced salt solution. In all experiments, the anti-sheep RBC (agglutination titer, 8000) was used at a dilution of 1:1000, e.g., 8 agglutinating units. The section rosette assay was performed with some minor modifications from the method that was described originally by Milgrom et al. (14). The wells of hanging drop depression slides were filled either with the 0.0% suspension of sensitized indicator cells (EA) or with unsensitized sheep RBC. A coverslip containing the tissue section was placed over the well, and the slide was inverted and incubated at 37°C for 30 min to allow the erythrocytes to settle onto the section and attachment to occur. The slide then was returned to the upright position and incubated for 30 min to allow unattached erythrocytes to drop back into the well. The attachment of EA was evaluated microscopically both quantitatively and qualitatively, and characteristic areas of the section were photographed.

**RESULTS**

**Controls.** MCA-induced tumors are composed of a complex mixture of malignant and host inflammatory cells, the composition of which changes as the tumor progresses. A variety of methods have been used by investigators to quantitate the different cell types. Therefore, it was imperative to document the reliability of the methods used in the present study. Granulocytes were recognized easily on a morphological basis alone. Cells with the morphology of lymphocytes also were quantitated easily in cytocentrifuged, stained cell preparations. Determination of the relative proportion of lymphocytes composed of T- and B-lymphocytes was more complex. In an effort to determine what proportion of the lymphocytes was composed of T-cells, suspensions from several tumors were tested by IIF with T-cell specific antiserum. More than 75% of the lymphocytes were T-cells (Table 1). The remainder of the cells that were counted as lymphocytes were assumed to be either B-lymphocytes or as yet undefined lymphocyte-like cells (15). Independent corroborations through specific B-cell quantitation was not possible because surface immunoglobulin was removed by enzyme digestion, and we do not have a specific B-cell antiserum. Macrophages were quantitated by virtue of their expression of IgG Fc receptors (EA rosette formation). Previous studies have established that IgG Fc receptor-positive cells in tumor suspensions are macrophages, regardless of whether they are actively phagocytic (18, 26, 28). In the present study the rosetted cells were cytocentrifuged routinely and examined morphologically. Greater than 90% were easily identifiable as macrophages (Fig. 1), a few were granulocytes, and a small proportion resembled lymphocytes. Reliable enumeration of tumor cells was not possible because they were not easily distinguished from macrophages on a morphological basis alone. However, because lymphocytes, granulocytes, and macrophages were accounted for by the various individual assays, it was assumed that most, if not all, of the remaining cells were neoplastic.

IIF assays on tumor sections with anti-T-lymphocyte and antimacrophage sera were controlled in the following manner. Normal rabbit serum was not reactive with the cells in tumor tissue sections (Fig. 2). FITC-goat anti-rabbit γ-globulin alone consistently produced negative results. The anti-T-cell serum produced strongly positive immunofluorescence on thymus but failed to stain liver or lung. The immunofluorescence reaction of anti-T on thymus and selected tumors was completely removed by absorption with thymus homogenate. The antimacrophage serum failed to react with thymus but clearly detected Kupffer cells in liver sections and alveolar macrophages in lung sections (Fig. 3). Furthermore, very distinct staining patterns were observed with the antisera on all tumors. There could be no doubt that they were outlining different cell populations. For example, when sequential sections of a high-macrophage, low-T-cell-content tumor were stained with antimacrophage serum, there was extensive evidence of macrophage infiltration (Fig. 4), but the T-cell section produced little evidence of T-cells (Fig. 5). The fluorescence patterns

### Table 1

**Detection of T-lymphocytes in tumor cell suspensions by IIF**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>% lymphocytes by morphology</th>
<th>% T-lymphocytes by immunofluorescence</th>
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<tbody>
<tr>
<td>B</td>
<td>25</td>
<td>20</td>
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<tr>
<td>GG</td>
<td>11</td>
<td>9</td>
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<tr>
<td>RR</td>
<td>17</td>
<td>13</td>
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<td>CCC</td>
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**a** At least 400 total cells were counted following cytocentrifugation and staining of tumor cell suspensions.

**b** IIF was performed on tumor cell suspensions with specific rabbit anti-T-lymphocyte serum. Normal rabbit serum produced membrane staining on less than 1% of tumor-associated cells.
that were produced with the various primary tumors with antimacrophage serum always were paralleled by the EA adsorption patterns. For example, high-macrophage-content tumors exhibited strong, diffuse EA adsorption over the entire tumor, while other tumors that contained only small foci of immunofluorescent positive cells also exhibited focal EA adsorption. Thus, macrophage distribution as determined by immunofluorescence was identical with the distribution of IgG Fc receptor-positive cells in the tumors. In contrast, fluorescence patterns produced by the anti-T-cell serum were not paralleled by EA adsorption patterns. Neither the antimacrophage nor the anti-T-cell serum reacted with normal mouse serum in gel diffusion analysis. Thus, neither serum detected tumor-associated immunoglobulin.

One of the major problems with cellular analyses of tumors is that the enzyme may produce biased release of certain cell types from the tumor. With respect to the T-lymphocytes and macrophages, that problem was controlled in the present study by comparing the results of suspension analyses with the results of IIF with specific cell antisera on the intact tumor. Although the IIF was semiquantitative at best, it did demonstrate that with few exceptions there was a close correlation between representation of T-cells and macrophages in the derived cell suspensions and in the intact tumor. For example, one tumor with a macrophage content of 17% contained sparsely distributed macrophages by IIF (1 to 2 + ), while a second tumor containing 54% macrophages was heavily infiltrated with macrophages (3 to 4 + ). A similar relationship was observed in most tumors that were studied. The comparisons between T-cell IIF and lymphocyte percentages in cell suspensions are presented below in the relevant sections on tumor analysis.

**Cellular Analysis of Small Primary MCA-Induced Tumors.** The surgically removed primary tumors were divided into 3 groups on the basis of size. The first group had a volume range of 0.06 to 0.23 cu cm. They all were removed with 2 to 3 days of their appearance as palpable tumors. Data from the cellular analysis of these tumors are presented in Chart 1. In that group, extremely disparate cellular pictures were observed. Three of the tumor cell suspensions were composed almost entirely of small lymphocytes, while macrophages and granulocytes represented less than 5% of the total cells. Immunohistological analysis of each of those tumors with T-cell antiserum revealed that they contained an extremely high concentration of T-lymphocytes and that the T-lymphocytes had completely infiltrated the tumor (Fig. 6). The immunohistological findings demonstrated (a) that the lymphocytes in the suspensions were predominantly T-cells and (b) that those high lymphocyte proportions in the cell suspensions were not an artifact of the enzymatic digestion. IIF analysis of the same three tumors with antimacrophage serum revealed that low but significant numbers of macrophages were present. The demonstration of significant numbers of macrophages in those tumors was confirmed by EA adsorption. Thus, IgG Fc receptor-positive cells were demonstrated throughout those tumors by virtue of their ability to absorb the antibody-sensitized erythrocytes.

The remaining five tumors in the small-size group each contained a lower relative proportion of lymphocytes (range, 10 to 35% of the tumor-associated cells), and all contained significant numbers of macrophages (range, 20 to 45%) and granulocytes (4 to 16%). The T-lymphocytes in those remaining small tumors also were distributed throughout the tumor mass, but there were fewer of them. Each of those tumors was infiltrated by significant macrophages as measured by IIF on sections.

Although the phagocytic capacity of macrophages should not be construed as an indicator of their antitumor activity, it does serve as a measure of their functional activity. Thus, yeast phagocytosis was performed on each of the tumors. The phagocytic percentage was a measure of the numbers of potentially phagocytic cells (macrophages and granulocytes) that actually ingested yeast. In the small tumor group, the phagocytic activity in those tumors that contained significant numbers of macrophages and granulocytes was low (mean phagocytic percentage, 12.6; range, 0 to 31%). The phagocytic percentage in the 3 tumors with low numbers of phagocytic cells was not considered to be reliable although the data are included in Chart 1.

**Cellular Analysis of Medium-sized Primary MCA-Induced Tumors.** The second group of primary tumors on which detailed cellular analysis was performed had a size range from 0.25 to 1.0 cu cm. They were removed between 3 and 7 days following their appearance as palpable tumors. The smallest of the tumors was indistinguishable from the 3 tumors in the smaller size group that had high T-lymphocyte contents (Chart 2). The remaining tumors all contained significant numbers of macrophages; e.g., the relative number of macrophages in the 7 larger tumors ranged from 10 to 40%, and all contained significant numbers of lymphocytes (range, 10 to 44%; mean, 31%). Low numbers of granulocytes were present in each (range, 5 to 10%). The most striking fact about that group of tumors was that they expressed quite a high level of phagocytic activity, ranging from 15 to 100% with several tumors expressing a phagocytic percentage above 30 (mean for 7 tumors, 47%).

Most of the medium-sized tumors exhibited T-cell and
macrophage distribution patterns, as determined by IIF, that were similar to those of the smaller tumors; e.g., significant numbers of T-cells were present throughout the tumor mass, and macrophages also were present throughout although they appeared to be more concentrated near the tumor periphery. A few of the larger tumors expressed a somewhat different immunohistological pattern. T-Lymphocytes were sparse and were concentrated at the tumor periphery. Generally, they were present singly or in small groups (Fig. 7). Macrophages still were present throughout the tumor.

**Cellular Analysis of Large Primary MCA-Induced Tumors.** The last group contained tumors with volumes greater than 1.0 cm. These tumors had been allowed to progress for at least 8 days after original palpation. Although the tumors in that group were large, they would have progressed to significantly greater volumes had they not been excised. For example, some tumors that were not removed reached volumes in excess of 4.0 cm before death of the animal. The macrophage contents of these tumors (Chart 3) were significantly higher than those of the tumors in the other 2 groups. Thus, the relative proportion of the cellular contents of the tumors in Group 3 that was composed of macrophages ranged from 35 to 56%. Also, somewhat higher numbers of granulocytes were observed. The relative number of lymphocytes that were present in the large tumors was considerably lower than in smaller tumors. Four of the 6 tumors contained only 10 to 15% lymphocytes, while the remaining 2 tumors contained 25%. Probably, this result did not represent an absolute decrease in T-cells because there had been at least a 2-fold increase in tumor size. The phagocytic activity of the macrophages and granulocytes also was decreased significantly in the large tumors as compared to the level that was observed with medium-sized tumors; e.g., the mean phagocytic percentage was 25% as compared to the mean level of 47% that was observed with medium-sized tumors.

The large tumors were relatively easily characterized by immunohistological techniques. T-Lymphocytes were very sparse and generally were observed near the tumor periphery. Macrophages had extensively infiltrated all of the tumors. IIF with antimacrophage serum demonstrated large numbers of fluorescent cells throughout the tumor; furthermore, strong homogeneous EA adsorption was observed over the entire tumor surface with each of the large tumors. It was evident from both EA adsorption and IIF that macrophages were intimately associated with tumor cells throughout the tumors.

A similar type of analysis was performed on over 50 primary tumors. Data from 22 of those tumors were presented for simplicity because the results from the remaining tumors were similar; i.e., the 22 tumors that were selected were representative of the data distribution in the 3 size groups.

**Immunohistological Analysis of Entire Tumors.** A major inherent problem in the foregoing study was that an attempt was made to perform several types of analyses on a single tumor. Thus, tumors were divided for suspension and histological analysis, and even though care was taken to remove representative portions of the tumor for IIF analysis, only a small portion was available. Therefore, it was not possible precisely to define cell distribution throughout the tumor. It would be optimal to analyze cross-sections of the entire tumor, because the question of cell distribution in the tumor is a critical one. To do this, we sectioned several tumors that had been frozen in toto. Cross-sections were obtained after cutting about half-way into the tumor. Thus, all areas, peripheral and central, were completely represented.

Because there was little apparent difficulty with T-lymphocyte distribution, e.g., large numbers were present in small tumors, but few were seen in large tumors, the present distribution analyses were confined to the macrophages. Sections from 7 different-sized tumors were analyzed for quantity and distribution of macrophages by IIF (Table 2). The results with the 7 tumors were very simple. Macrophages were distributed throughout each tumor but were more concentrated in peripheral areas. Also, some of the tumors appeared to have very high numbers of tumor-associated macrophages, while others had relatively fewer. For example, Tumor 382 contained very few macrophages.
in central areas (Fig. 8) and moderate numbers in peripheral areas (Fig. 9), while Tumor 425 contained moderate numbers of macrophages in central areas (Fig. 10) and heavy infiltration in peripheral regions of the tumor (Fig. 11). It was very easy to distinguish positive from negative cells when the macrophages were not present in large numbers (Figs. 8 to 10), but when an area of the tumor was heavily infiltrated it was difficult to distinguish positive from negative cells (Fig. 11). Macrophages were most striking when they occurred in connective tissue surrounding a tumor (Fig. 12) because there was a reduced level of background staining.

**DISCUSSION**

One of the major problems with the cellular analysis of tumors has been the almost complete lack of quality control. Each of the groups that has been involved in this type of investigation has used some form of enzyme digestion, generally involving trypsin (2-4, 6-11, 16, 21, 23, 25, 26, 28). Despite universal recognition of the potential problem of preferential cell release by enzyme digestion, no good method has been devised to evaluate whether the cell populations derived by the digestion procedure were representative of the cellular composition of a tumor. Recently, Russell et al. (20) suggested that it would be valuable to quantitate cell yield. In fact, cell yield determination provides no information about population selection. It does not matter whether 5 or 80% of the cells are recovered during the digestion process. The exception occurred with very small tumors that contained macrophages by immunofluorescence and EA adsorption analysis but produced cell suspensions containing very few macrophages. The macrophages may have been selectively destroyed by the enzyme, but it is also possible that because low cell numbers were derived from the small tumors the few macrophages present simply adhered to the glass surfaces during trypsinization and washing.

It is important in the interpretation of the results of the present study to understand the effect of tumor heterogeneity. Because chemically induced tumors vary in their immunogenicity and possess individually distinct antigens, one might expect that the cellular response to those tumors would be heterogeneous with respect to how rapidly it develops and with respect to its strength. Thus, the fact that very disparate cellular pictures were observed, especially with smaller tumors, was predictable because each cellular analysis represented a single point on the continuum that was the cellular response to a single tumor. Clearly, it is nearly impossible to perform an accurate characterization of cellular responses to tumors in general by performing such single-point analyses of primary tumors even when large numbers of tumors are included in the study. The major reason for performing such a study was to obtain enough information on primary tumors that one would be able to say that results derived from similar analyses of passaged tumors were not artifacts caused by the conditions of tumor passage. For example, when an initially high T-cell response was observed with a passaged tumor (27), was it an artifact of passaging or does a similar response occur within primary tumors?

Several important observations were made during this study of cellular activity in primary chemically induced fibrosarcomas. Our results are in agreement with others to the extent that large progressing tumors contained few T-lymphocytes within the tumor mass (12). Furthermore, we have demonstrated for the first time that the same tumors were infiltrated by large numbers of T-cells very early in their development. Previously, high numbers of T-cells have been documented within the tumor mass only during tumor rejection (12). The significant decrease in the relative numbers of T-cells within the tumor that occurred as the tumors increased in size paralleled the timing of systemic immunosuppression that has been reported previously to occur with progressive tumor growth (1). It may be that the lymphocyte decreases within the tumor are the tumor-associated manifestation of the generalized immunosuppression.

Significant numbers of macrophages were found in all tumors, a fact that has been reported previously for primary chemically induced tumors (18). Recent studies have demonstrated a suppressive effect on T-cell function by acti-
vated macrophages (24) and have demonstrated that systemic immunosuppression in tumor-bearing animals is in part due to macrophages (13). An attractive speculation would be that the tumor-associated macrophages are at least in part responsible for suppression of the tumor-associated immune response, thus facilitating tumor progression.

Two observations were made on the macrophage population that had not been made in previous studies. First, small tumors tended to have fewer macrophages than large ones, although that could have been an artifact of the methodology because significant numbers of the adherent macrophages could have been lost during isolation of single-cell suspensions. Second, functional activity of the macrophage population as measured by yeast phagocytosis was low initially, rose to significant levels in medium tumors, and decreased in large tumors. The decreased functional activity that occurred with increased tumor size paralleled the decreases in T-cell numbers in the tumors. It is possible that a direct correlation was involved; i.e., decreased T-cells resulted in decreased macrophage activation. Alternatively, decreased macrophage function could represent changes in the infiltrating macrophage population; e.g., a higher proportion of infiltrating immature cells (monocytes) in larger tumors might result in decreased overall function.

The immunohistological analyses of macrophage distribution were extremely important. They represent the first clear documentation of the fact that macrophages are distributed throughout the tumor mass. This opens up the highly attractive possibility that macrophages may be able to be objectively identified and quantified in tumor sections. If antihuman macrophage sera became generally available, it would be possible to identify and localize macrophages precisely in human tumors, a technique that could prove to be extremely helpful clinically. The observation that macrophages usually were slightly more concentrated in peripheral regions of the tumor suggested that those cells reached the tumors by migration from surrounding tissue. Little value would be gained by speculating further on the results. Clearly, much remains to be learned about the functional dynamics of potential immune effector cells that infiltrate antigenic tumors and about the effect these cells actually may have on spontaneous progressive neoplasms.

REFERENCES


Fig. 1. Tumor suspensions were rosetted with EA, incubated at 37° for 60 min to allow erythrophagocytosis, cytocentrifuged, and stained. a, 2 rosette-positive, phagocytic macrophages (M), a rosette-negative tumor cell (T), and a lymphocyte; b, 2 rosette-positive macrophages, 1 phagocytic (PM) and 1 nonphagocytic (NPM), and a rosette-negative tumor cell (T). × 480.

Fig. 2. Primary MCA-induced tumor stained by IIF with normal rabbit serum. × 400.

Fig. 3. Murine alveolar macrophages stained by IIF with rabbit anti-mouse macrophage serum. × 400.

Fig. 4. A primary MCA-induced tumor (same as in Fig. 4) stained by IIF with rabbit anti-mouse macrophage serum. × 400.

Fig. 5. A primary MCA-induced tumor stained by IIF with rabbit anti-T-lymphocyte serum. × 400.

Fig. 6. A small primary MCA-induced tumor stained by IIF with anti-mouse T-cell serum. × 160.
Fig. 7. A large primary MCA-induced tumor stained by IIF with anti-mouse T-cell serum. × 400.
Fig. 8. Primary MCA-induced Tumor 382 stained in a central area by IIF with anti-mouse macrophage serum. × 400.
Fig. 9. Primary MCA-induced Tumor 382 stained in a peripheral area by IIF with anti-mouse macrophage serum. × 400.
Fig. 10. Primary MCA-induced Tumor 425 stained in a central area by IIF with anti-mouse macrophage serum. × 400.
Fig. 11. Primary MCA-induced Tumor 425 stained in a peripheral area by IIF with anti-mouse macrophage serum. × 400.
Fig. 12. Macrophages (positively stained) and tumor cells (negatively stained) in loose connective tissue surrounding Tumor 408. IIF with anti-mouse macrophage serum. × 400.
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