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Quantitative Assessment of the Leukocyte Infiltrate in Ovarian Cancer and Its Relationship to the Expression of C-C Chemokines

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We have defined the host leukocyte infiltrate in epithelial ovarian tumors and related this to the expression of C-C chemokines. Immunohistochemical analysis of 20 paraffin-embedded biopsies showed that the infiltrate was primarily composed of CD68$^+$ macrophages and CD8$^+$/CD45RO$^+$ T cells (median values, 3700 cells/mm^3 and 2200 cells/mm^3, respectively). Natural killer cells, B cells, and mast cells occurred in lower numbers (median values, 0 to 200 cells/mm^3). Eosinophils were rarely seen and neutrophils were mainly confined to blood vessels. More infiltrating cells were found in stromal than in tumor areas. Only macrophages occurred in significant numbers in areas of necrosis ($P < 0.0005$). Using in situ hybridization to mRNA, we examined expression of the chemokines MCP-1, MIP-1α, MIP-1β, and RANTES. MCP-1 and MIP-1α were expressed by significantly more cells than MIP-1β and RANTES ($P < 0.005$). In tumor epithelial areas, the predominant chemokine was MCP-1. MCP-1 and MIP-1α were the predominant stromal chemokines. A significant correlation was found between the total number of CD8$^+$ T cells and the number of cells expressing MCP-1 ($r_s = 0.63$ and $P < 0.003$, respectively) and between the CD8$^+$ population and RANTES-expressing cells ($r_s = 0.6$ and $P < 0.003$). A correlation was also found between CD68$^+$ macrophages and the number of cells expressing MCP-1 ($r_s = 0.50$ and $P = 0.026$). We suggest that MCP-1 may be responsible for the leukocyte infiltrate in ovarian carcinomas, but the expression of other chemokines may determine its exact nature. (Am J Pathol 1997, 150:1723–1734)

Many solid tumors of epithelial origin contain a significant number of host leukocytes, mainly macrophages and lymphocytes. Even before the introduction of specific monoclonal antibodies, analysis of a variety of tumors, including carcinoma of the colon, lung, breast, and stomach, demonstrated the presence of such cells, particularly in the stroma.[1] These infiltrating cells have recently been described in detail in carcinoma of the breast and dysgerminoma of the ovary using immunohistochemistry with monoclonal antibodies in paraffin-embedded material.[2,3] They have also been defined in frozen sections in a variety of other solid tumors.[4,5] Previous attempts to characterize the infiltrate in carcinoma of the ovary included collagenase digestion of whole tumor followed by sedimentation-velocity separation of inflammatory cells[6] and analysis of frozen tumor sections with monoclonal antibodies.[7] However, these studies provided conflicting results; in the former, the infiltrate was found to consist of both macrophages and T cells, whereas in the latter, only T cells were described in significant numbers, and these were mainly CD4$^+$.

The concept of a macrophage balance was introduced by Mantovani to encapsulate the notion that macrophages may aid or inhibit tumor growth according to their state of activation.[8] Under normal conditions, macrophage-derived factors such as tumor necrosis factor-α, platelet-derived growth factor, and transforming growth factor-β may promote growth,[9] for instance, by encouraging neovascularization and stroma formation. Furthermore, some products of macrophage activation may cause DNA damage in rapidly cycling cell populations,[10] thereby promoting tumor progression. However, macrophage activation may

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also lead to tumor cell destruction. This can be induced in experimental animal models[11] and could account for the effects seen with adjuvant tumor therapies, such as Calmette-Guérin bacillus. Increasing the macrophage to tumor cell ratio may also lead to tumor cell destruction *in vitro*.[12] Furthermore Chinese hamster ovary cells transfected with monocyte chemoattractant-1 (MCP-1) fail to grow in syngeneic hosts and contain a mononuclear infiltrate.[13] These effects may be mediated by products of macrophage activation.[14] The idea of the macrophage balance may extend to other tumor-infiltrating leukocytes, particularly T lymphocytes.

Although the role of infiltrating cells in epithelial tumors is controversial, a likely stimulus for their presence is the local production of chemokines. These are small (8 to 10 kd) cytokines that are chemoattractant for a variety of leukocytes and other cells. They are generally classified on the basis of two conserved amino-terminal cysteine residues that may either be adjacent, C-C, or separated by another amino acid, C-X-C. The C-C chemokines are active on T cells and monocyte/macrophages, but C-X-C chemokines are more specific for neutrophils. The leukocyte content of a tumor may depend on the chemokines that are expressed. In a previous study, we described the production of MCP-1 by human epithelial ovarian tumor cells and demonstrated the presence of a chemokine gradient between ascites and plasma.[15] MCP-1 has been found in other tumors, such as malignant glioma,[16] where macrophages are plentiful. Although the presence of this chemokine may explain the macrophage infiltrate in ovarian tumors, there are no data that correlate the level of MCP-1 expression and the extent of the infiltrate in individual tumors. Moreover, the contribution of other chemokines has not been assessed. To investigate the relationship between host immune cells, chemokines, and tumor in epithelial ovarian cancer, we have defined quantitatively the nature and distribution of the leukocyte infiltrate using a panel of antibodies in paraffin-embedded material. We also analyzed cryostat sections of epithelial tumors for expression of the C-C chemokines macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES (regulated upon activation, normal T cell expressed and stimulated) and compared these with MCP-1 expression in the same samples. The number of chemokine-expressing cells was then correlated with the infiltrating cell population.

Materials and Methods

Tissue Samples

Twenty human epithelial ovarian tumors were used to assess the infiltrating leukocyte population. Nine were collected at the time of operation and were fixed in formol saline for 24 hours. Eleven were obtained from the Hammersmith Hospital (London, UK) archives. There were 2 borderline tumors, 15 papillary serous cystadenocarcinomas (5 grade 1, 5 grade 2, and 5 grade 3), 2 papillary endometrioid tumors (grade 2), and 1 solid endometrioid/undifferentiated tumor (grade 3). All tissue samples were paraffin embedded before cutting 4-μm sections onto silane-coated slides. The histological type and grade of all specimens was reassessed at the time of the study, using criteria previously outlined by us.[17]

An additional 20 frozen epithelial tumors were assessed for C-C chemokine expression by *in situ* hybridization. Ten of these tumors were from the same patients as the paraffin-embedded material and ten samples were obtained from the Queen's Hospital, Belfast. One was classified as a borderline/papillary serous cystadenocarcinoma (grade 1); eleven were papillary serous cystadenocarcinomas (three grade 1, four grade 2, and four grade 3); two were mucinous cystadenocarcinomas (grade 2); three were papillary endometrioid carcinomas (grade 2); one was a papillary serous adenocarcinoma (grade 3), and two were serous solid/cystic adenocarcinomas (grade 3). The 6-μm cryostat sections were cut onto 3-aminopropyl-triethoxy-silane-coated four-well slides. These sections were also used to assess the $CD68^+$ and $CD8^+$ population. Sections were air dried for 1 hour and fixed in 4% paraformaldehyde for 20 minutes or formol saline for 5 minutes, respectively, before immunostaining.

Antibodies

A panel of 11 antibodies was used to phenotype the infiltrating leukocyte population: anti-CD3 (DAKO, High Wycombe, UK) for all T lymphocytes, anti-CD8 (C8/144B, DAKO) for cytotoxic T lymphocytes in paraffin-embedded material, anti-CD8 (DK125, DAKO) for cytotoxic T lymphocytes in frozen material, anti-CD45RO (UCHL1, DAKO) for memory T lymphocytes, anti-CD4 (MT310, DAKO) for helper T lymphocytes in frozen material, anti-CD68 (PG-M1, DAKO) for macrophages in paraffin-embedded material, anti-CD68 (EBM 111, DAKO) for macrophages in frozen material, anti-CD20 (L26, DAKO) for all B lymphocytes, anti-CD57 (NK1, DAKO) for natural killer cells, anti-mast-cell-tryptase (AA1, DAKO) for mast cells, and MAC 387 (DAKO) for granulocytes. The presence of eosinophils was determined in hematoxylin-and-eosin-stained sections according to their characteristic staining and morphology.

Immunohistochemistry

A three-step indirect streptavidin-peroxidase system was used throughout. Immunostaining was revealed by the chromogen 3,5-diaminobenzidine (DAB). All tissue sections were treated with H_2O_2 to block endogenous peroxidase activity before staining. In addition, sections stained with anti-CD3 were blocked with normal swine serum (DAKO) before the primary antibody was applied. Sections were incubated sequentially with primary antibody, biotinylated rabbit anti-mouse IgG (DAKO), and streptavidin-peroxidase conjugate (DAKO) for 35 minutes each. Between each step, sections were washed twice in fresh phosphate-buffered saline, pH 7.2, for 5 minutes. The final incubation with DAB was for a maximum of 2 minutes, followed by a 2-minute wash in water. Sections were counterstained with Harris' hematoxylin for 30 seconds. Positive controls were obtained by staining sections of tonsil, spleen, or stomach as appropriate. Omission of the primary antibody was used to provide negative controls.

Assessment of Cell Numbers and Distribution

All cell counts were performed using a Nikon Labophot II microscope (Nikon, Kingston, UK) at a magnification of ×200 (20× objective and 10× eyepiece). Only cells with cytoplasmic staining, nuclear counterstain, and appropriate morphology were included. The area counted in each section was chosen randomly from a representative field of tumor. Cell counts were performed with the aid of a 10 × 10 index grid (Graticules, Tonbridge, UK). For each section, nine areas were assessed with the grid arranged in a 3 × 3 pattern, to give a total area of 2.3 mm^2.

Area counts were carried out using a modified Chalkley array (Graticules) over the same area as the cell counts. The modified Chalkley array consists of 25 randomly arranged points; the proportion of points coincident with any tissue component is proportional to its area and volume fractions.[18] In addition to total cell counts, four different components within the tumors were assessed: tumor cell islands, stroma, areas of necrosis, and areas of space. Areas of necrosis were defined as having cell debris, apoptotic bodies, or red cell fragments. Areas of space included both real spaces, for instance, between tumor papillae, and those created by fixation and processing.

The method of DeHoff and Rhines[19] as described by Aherne and Dunhill[20] was used to estimate the number of cells per mm^3. Equivalent nuclear diameters for each cell type were determined by tracing a minimum of 100 cell circumferences using an image analysis program (Lucia, Nikon, UK) and a 100× objective oil immersion lens. A frequency histogram of equivalent diameters was constructed for each cell type. The left-hand tail of each histogram was estimated to correct for optically lost caps. The mean tangent diameter (D) was then calculated from the mean of the set of equivalent diameters (d), according to the formula $D = 4d/\pi$. Estimates of equivalent diameters derived from cell circumference tracing were validated by assessing the mean diameter of 40 erythrocytes contained within the sections (mean = 6.61 μm, standard deviation = 0.66 μm).

Cell counts were combined with area counts to produce an estimate of the number of cells within each tumor compartment/mm^2. This was then converted to an estimate of cells/mm^3 using the formula $N_v = N_A/(D + t)$, where N_v = number of cells/mm^3, N_A = number of cells/mm^2, D = mean tangent diameter, and t = section thickness, taken to be 4 μm.

In Situ *Hybridization and Assessment of Chemokine Message Expression*

All the cDNAs used in the these studies were the kind gift of Professor Alberto Mantovani (Mario Negri Institute, Milan, Italy), other than β-actin, which was obtained from Dr. L. Kedes (Stanford University, Stanford, CA). cDNAs for MCP-1, MIP-1α, MIP-1β, and RANTES were subcloned into suitable transcription vectors before generating ^{35}S-labeled sense and antisense riboprobes as previously described.[15] *In situ* hybridization was performed according to our standard protocol and incubation was at 4°C for 10 days. Message expression was seen in all antisense-probed sections but in none of the sense control sections. All sections were positive for the expression of β-actin.

To compare the expression of different chemokine mRNAs, the total number of positive cells were counted in 10 adjacent high-power fields (40× objective and 10× eyepiece), giving a total area of 0.73 mm^2. Fields were chosen to include at least one area of high expression but to avoid the edge of the section and areas of artifact. The proportion of tumor and stroma was assessed over the same area using the modified Chalkley array. Due to the difficulty in determining the equivalent of the mean nuclear diameter, cell counts were expressed as cells/mm^2.

Immunohistochemistry and Cell Counts in Frozen Sections

Paraformaldehyde- or formalin-fixed frozen sections were stained for $CD68^+$ macrophages and $CD8^+$ T cells with the antibodies EBM111 and DK125, respectively. $CD4^+$ T cells were detected in paraformaldehyde-fixed frozen sections using the antibody MT310. The staining procedure was otherwise identical to that for paraffin-embedded sections. Counterstaining was performed with 1% toluidine blue. Cells were counted in 10 randomly chosen high-power fields (40× objective and 10× eyepiece; total area, 0.73 mm^2) and were expressed as cells/mm^2.

Statistical Analysis

As the data were not normally distributed, medians were used to summarize the data, and nonparametric methods of analysis were used to calculate *P* values. Wilcoxon's matched pairs signed rank test was used for all comparisons between matched pairs of data. Spearman's rank correlation was used to determine the relationship between the $CD3^+$, $CD8^+$, and $CD45RO^+$ T cell populations and to test whether a correlation existed between the number of chemokine-expressing cells and the number of $CD68^+$ or $CD8^+$ cells. The Mann-Whitney *U* test was used to test whether there was a significant difference between the number of $CD68^+$ and $CD8^+$ cells in frozen and paraffin sections and also to assess the relationships between the leukocyte populations, chemokines, and tumor grade.[21] Within each analysis, up to 10 comparisons were made. Using the Bonferroni method to correct for multiple comparisons,[22] only *P* values <0.005 were considered to be statistically significant. However, as this method is highly conservative, *P* values <0.05 are also shown.

Results

Composition and Localization of the Cellular Infiltrate in Epithelial Ovarian Cancer

The nature of the cellular infiltrate was assessed in 20 epithelial carcinomas. For each cell type, 9 high-power fields were counted at a magnification of ×200 (20× objective and 10× eyepiece). After correcting for the mean tangent diameter, cell numbers were expressed as cell densities (cells/mm^3), both for the total area assessed and for each of the four different compartments within the tumor. These were tumor cell islands, stroma, necrotic areas (defined by the presence of cell debris, apoptotic bodies, or red cell fragments) and areas of space between these components. The proportion of whole tumor occupied by each of these compartments is given in Table 1. The volume of whole tumor occupied by tumor cells and stroma were similar (43 and 37%, respectively). Necrosis accounted for 4% of the volume of the tumor, and that occupied by real and artifactual space was 16%. There were no significant differences between the volumes occupied by each compartment with tumor grade. Two patterns of infiltrate were distinguishable. Cells present in large numbers were $CD68^+$ macrophages, $CD3^+$, $CD8^+$, and $CD45RO^+$ T cells, whereas those found in much lower numbers were B cells, natural killer (NK) cells, and mast cells (Figure 1a). Table 2 gives the median and range for each of the different cell types per mm^3 of total tumor. There was a significant difference between those cells present in high ($CD3^+$, $CD8^+$, and $CD45RO^+$ T cells and $CD68^+$ macrophages) and low ($CD20^+$ B cells, $CD57^+$ NK cells, and mast cells) numbers ($P < 0.0005$) but not between the cell types within each group. In general, the infiltrating cell density was significantly higher in the stroma compared with the tumor compartment (Figure 1, b and c). For instance, the median value for the number of $CD3^+$ T cells found within the stroma was 3800 cells/mm^3 compared with 1600 cells/mm^3 in the tumor cell areas ($P < 0.005$). Similar values were obtained for $CD45RO^+$ T cells. Median values for $CD68^+$ macrophages were 6700 cells/mm^3 and 2100 cells/mm^3 in stroma and tumor areas, respectively ($P < 0.05$). The exception to this was the $CD8^+$ population in which there was no significant difference between the numbers found within the stroma and the tumor cell islands (median values, 4300 cells/mm^3 and 2500 cells/mm^3, respectively; $P = 0.083$). In the stroma, T cells occurred singly or in clusters (Figure 2a), but when associated with tumor cell islands, they were only found alone (Figure 2b). The most striking difference in cell density was seen in regions of necrosis, where a significantly greater number of macrophages were observed than any other cell type ($P < 0.0005$, for all comparisons; median, 12,700 cells/mm^3; range, 0 to 128,000 cells/mm^3; Figure 1d). Macrophages in these areas had an activated morphology with abundant, foamy cytoplasm (Figure 2c). As cells that

Table 1. *Proportion of Tumor Volume Occupied by Various Components (Means and Ranges)*

Tumor	Stroma	Necrosis	Space
43% (14–90%)	37% (8–82%)	4% (0–37%)	16% (0–54%)

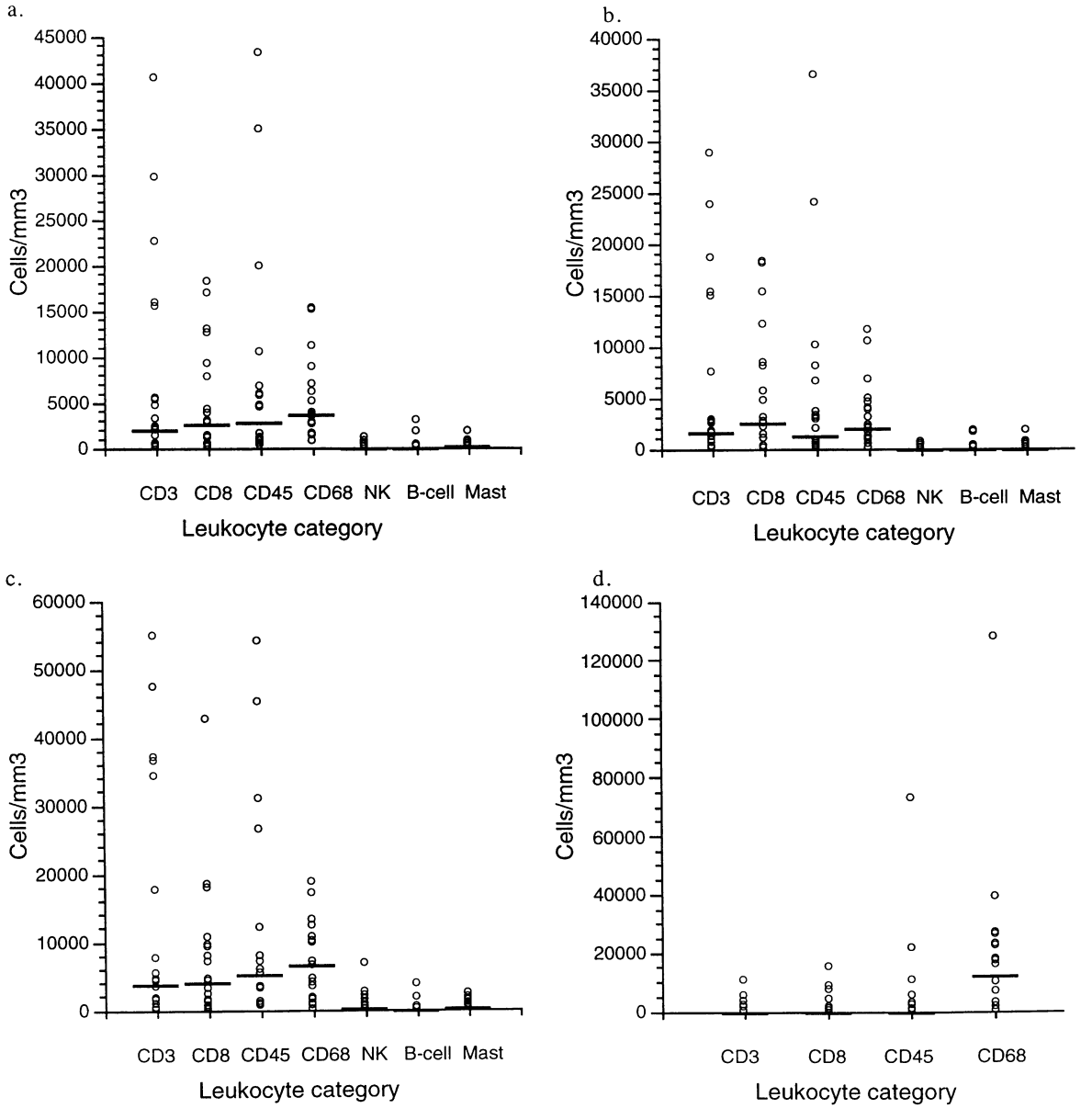

Figure 1. *Scatter plots demonstrating the absolute numbers of infiltrating leukocytes (cells/mm³) in whole tumor* (a), *tumor cell islands* (b), *and stromal* (c) *and necrotic* (d) *areas for each of the seven leukocyte markers examined. Only the T-cell subsets and CD68⁺ macrophages are shown in* d *for clarity. The* horizontal bars *represent median values.*

appeared effete were excluded from the counts, the total number of macrophages may have been underestimated. There was no correlation between the number of infiltrating $CD68^+$, $CD3^+$, $CD8^+$, or $CD45RO^+$ cells and tumor grade. Neutrophils were assessed using morphological criteria and the monoclonal antibody Mac 387. In contrast to the other cell types, they appeared to be largely confined to blood vessels. Although eosinophils were seen in occasional sections within the stroma and associated with tumor cells, they were rare. Neither neutrophils nor eosinophils were included in the cell counts.

Spearman's rank correlation was used to examine the relationship between the three T cell markers, both for total cell counts and for cells found within tumor cell areas and stroma. In all cases, there was a significant correlation between the three markers ($r_s > 0.7$ and $P < 0.005$). As there was no significant difference between the total number of $CD3^+$, $CD8^+$, or $CD45RO^+$ T cells, this suggests that the majority of infiltrating T cells have a cytotoxic memory phe-

Table 2. *Median Values and Ranges for Seven Tumor-Infiltrating Leukocyte Populations/mm³ of Total Tumor*

Cell type	Median	Range
$CD3^+$	2200	50–40,300
$CD8^+$	2800	0–18,200
$CD45RO^+$	2900	200–43,200
$CD68^+$	3700	600–15,200
NK cells	100	0–1,000
B cells	0	0–2,900
Mast cells	200	0–1,600

notype. There was no correlation between these T cell markers and the $CD68^+$ macrophage population. Staining for $CD4^+$ T cells was undertaken in frozen sections to determine their contribution to the total T cell infiltrate. However, there was cross-reactivity with other mononuclear cells, and as it was not always possible to discriminate accurately between the different cell types in these sections, $CD4^+$ cells were not included in the final assessment.

Chemokine Expression in Epithelial Cancer

The number of cells expressing each of the C-C chemokines MCP-1, MIP-1α, MIP-1β, and RANTES was determined in 10 high-power fields per cryostat section (40× objective and 10× eyepiece). Final cell counts were expressed per mm^2. Because the *in situ* hybridization studies were performed on cryostat sections counterstained with toluidine blue, areas of necrosis could not be reliably distinguished from artifact. Therefore, cell counts were assessed only for tumor or stromal areas. The total number of cells expressing MCP-1 or MIP-1α was significantly greater than the number expressing MIP-1β or RANTES ($P < 0.005$; Figure 3a). MCP-1 was expressed by more cells within tumor islands than any other chemokine ($P < 0.0005$; median value, 72.5 cells/mm^2; Figure 3b), but there was no significant difference between the number of MCP-1-expressing cells in tumor and stroma ($P < 0.03$). No difference was found in the number of MCP-1-expressing cells with grade. Within the stroma, the number of cells expressing MCP-1 and MIP-1α were similar (median values, 21 cells/mm^2 and 26 cells/mm^2, respectively). MIP-1α was expressed by more stromal cells than MIP-1β or RANTES ($P < 0.0005$; Figure 3c). MCP-1-expressing cells within the tumor islands tended to occur in discrete clusters (Figure 4a), whereas MCP-1- and MIP-1α-expressing cells in stroma occurred alone (Figure 4, b and c). The mean percentage of tumor cells expressing MCP-1 within a cluster was 8.2% (SD = 5.8%). In the stroma, the median number of cells expressing MIP-1β (Figure 4d) and RANTES were 2 cells/mm^2 and 7 cells/mm^2, respectively (Figure 3c). Although silver grains indicating MCP-1 expression were frequently associated with typical tumor cell nuclei, MIP-1α, MIP-1β, and RANTES were generally associated with smaller and more intensely stained nuclei, suggesting that they might be derived from the infiltrating leukocyte population.

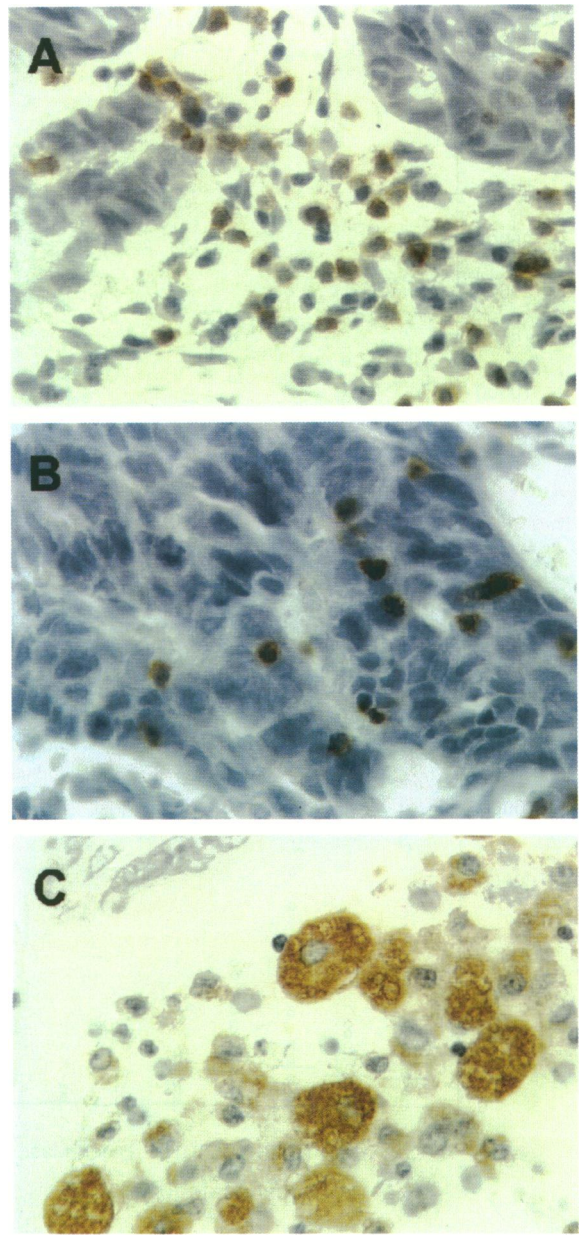

Figure 2. *T cells and macrophages within epithelial ovarian carcinomas. T cells were found either as clusters in stroma* (**A**) *or singly within both tumor* (**B**) *and stroma. Even when T cells were closely apposed to tumor cells, there was no evidence of apoptosis or necrosis. Macrophages were found at highest density in areas of necrosis* (**C**). *All immunostaining was revealed with the chromogen DAB and counterstained with Harris' hematoxylin. Magnification, × 700.*

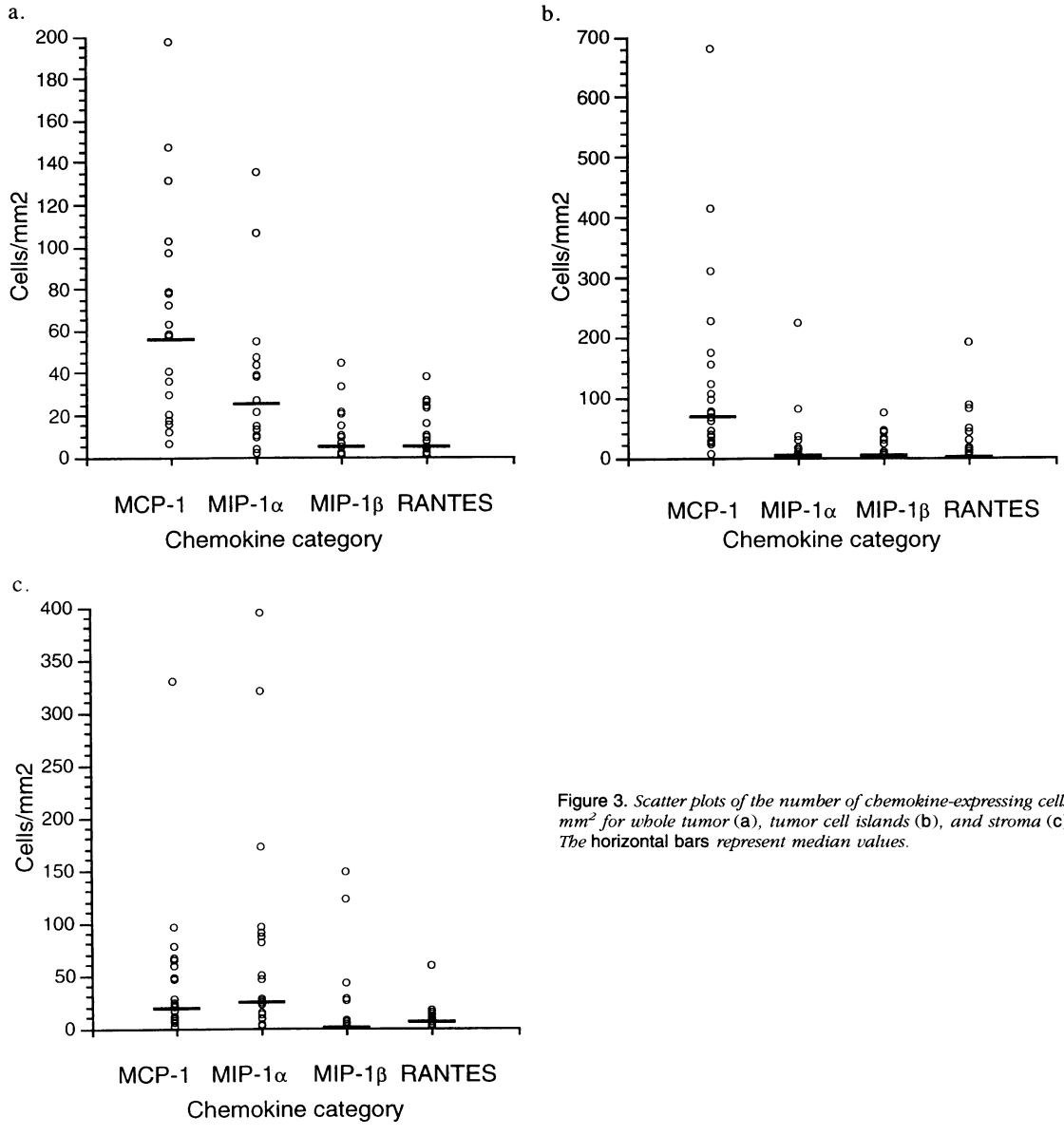

Figure 3. *Scatter plots of the number of chemokine-expressing cells/mm² for whole tumor* (a), *tumor cell islands* (b), *and stroma* (c). *The* horizontal bars *represent median values.*

Relationship between Chemokine Expression and Infiltrating Cells

$CD68^+$ macrophage and $CD8^+$ T cell counts were also assessed in cryostat sections to explore possible relationships between them and the number of chemokine-expressing cells. The antibody used to detect macrophages in frozen sections (EBM 111) was not as specific as that for paraffin-embedded material (PG-M1); therefore, only cells with positive cytoplasmic staining and characteristic nuclear morphology were included in the cell counts. No significant difference was found between the total number of macrophages or $CD8^+$ cells per mm^2 in the frozen and paraffin sections (data not shown). A possible correlation was found between the number of cells expressing MCP-1 and the number of $CD68^+$ cells ($r_s = 0.50$; $P = 0.026$). A significant correlation was found between the $CD8^+$ population and MCP-1 expression ($r_s = 0.63$; $P < 0.005$; Figure 5a) and the $CD8^+$ population and RANTES expression ($r_s = 0.6$; $P < 0.005$; Figure 5b). There were no significant correlations between the number of $CD68^+$ or $CD8^+$ cells and expression of the other chemokines.

Discussion

This is the first time that the nature and distribution of the leukocyte population in human epithelial ovarian cancer have been assessed quantitatively and com-

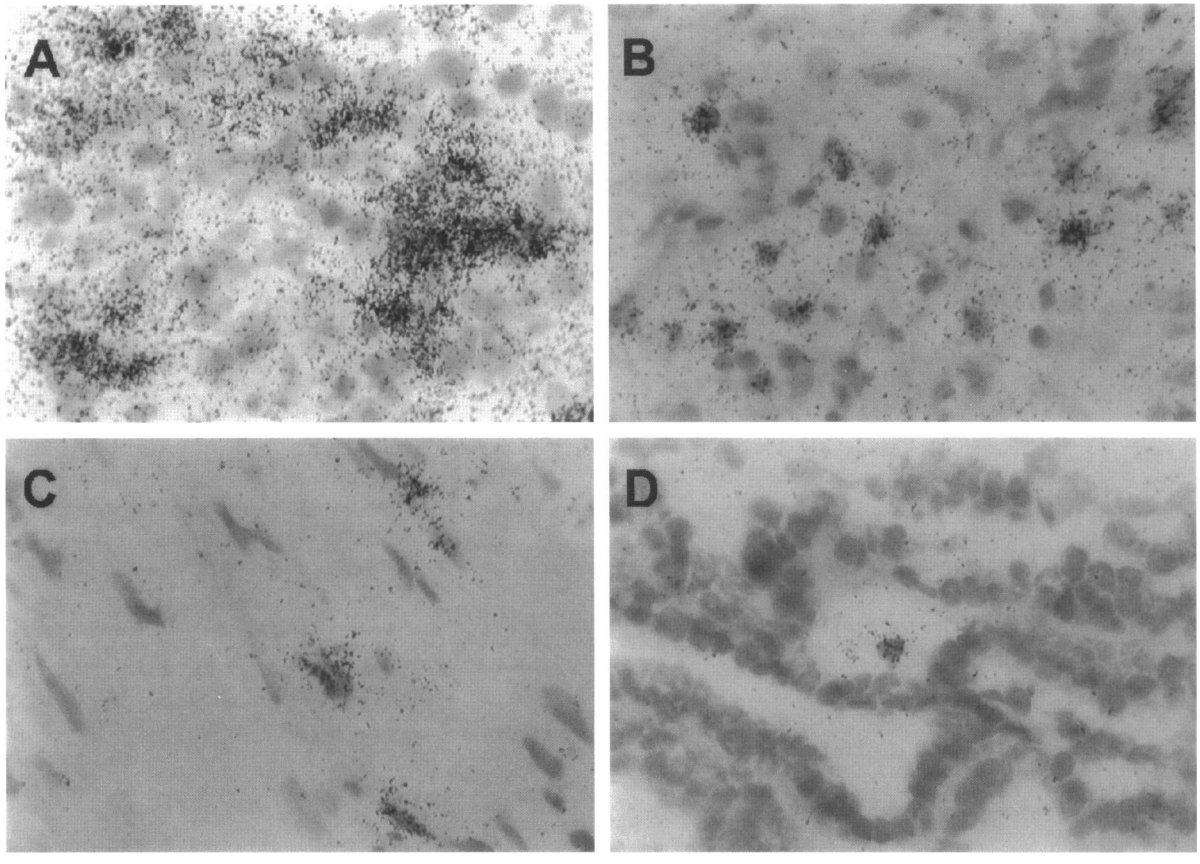

Figure 4. *Chemokine mRNA detected by isotopic in situ hybridization in frozen sections of ovarian carcinomas. MCP-1 expression was frequently clustered within tumor areas* (A), *whereas MIP-1α was clearly associated with single cells* (B). *MCP-1 was also expressed by large numbers of stromal cells* (C). *MIP-1β* (D) *and RANTES were infrequently expressed. All sections were counterstained with 1% toluidine blue. Magnification,* ×*700.*

pared with the expression of C-C chemokines. Furthermore, it is the first demonstration that the chemokines MIP-1α, MIP-1β, and RANTES are expressed in these tumors. We have shown that the host infiltrate consists largely of macrophages and T cells. The high degree of correlation between cells positive for the markers CD3, CD8, and CD45RO implies that the majority of infiltrating T cells have a cytotoxic memory phenotype. Other leukocytes such as B cells, NK cells, and mast cells were present in low numbers, whereas eosinophils were seen only occasionally and neutrophils appeared to be largely confined to blood vessels. More cells were found in the stroma, but both T cells, particularly $CD8^+$ cells, and macrophages were also found in close proximity to tumor cells. Whereas infiltrating cells in the stroma often occurred in aggregates, they were found alone much more frequently when associated with tumor cells. Only $CD68^+$ macrophages were found in high numbers in areas of necrosis, and here they appeared to have an activated morphology.

Three previous studies have specifically addressed the nature of the leukocyte infiltrate in ovarian cancer, but none of these has looked quantitatively at cell distribution. Haskill et al[6] used sedimentation-velocity to separate out the components of the inflammatory infiltrate in 38 epithelial ovarian tumors. They concluded that T cells and macrophages were the dominant components with a lesser contribution from B cells and NK cells. Kabawat et al[7] analyzed cryostat sections from a series of 70 tumors with a panel of monoclonal antibodies and concluded that very few macrophages were present but that the majority of the infiltrate consisted of $CD4^+$ T cells. Again, they found few B cells and NK cells. In the most recent study, again on cryostat sections, van Ravenswaay Claasen et al[23] concluded that the majority of infiltrating cells were macrophages and T cells expressing the $\alpha\beta$ T-cell receptor but that the $CD4^+/CD8^+$ ratio was difficult to determine because of the reactivity of anti-CD4 with macrophages. We were also unable to assess the number of $CD4^+$ cells in frozen sections due to cross-reactivity of the anti-CD4 antibody with other mononuclear cells.

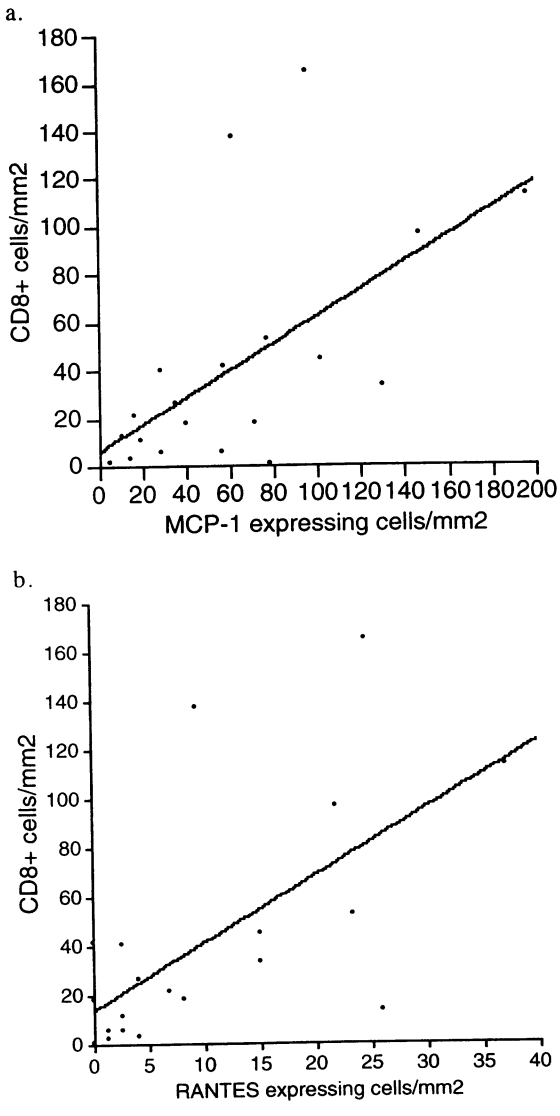

Figure 5. *Scatter plots demonstrating the relationship between the number of CD8$^+$ cells and the number of cells expressing MCP-1 mRNA* (**a**; $r_s = 0.63$ *and* $P < 0.005$) *and RANTES* (**b**; $r_s = 0.6$ *and* $P < 0.005$).

Our observations on epithelial ovarian carcinoma are supported by work in other carcinomas. Studies on both paraffin-embedded[2] and frozen specimens of breast cancers[24] support the contention that macrophages and T cells are the dominant infiltrating cells and that most of the T cells are CD8$^+$.[24] An assessment of the infiltrating cell population in seven cases of dysgerminoma of the ovary again found that tumor-infiltrating lymphocytes were predominantly CD8$^+$ and most of the intratumor T cells expressed the $\alpha\beta$ T-cell receptor.[3] Even before the introduction of monoclonal antibodies, studies by Svennevig et al[1,25] showed that T cells and macrophages predominate in a variety of carcinomas. They also found macrophages in large numbers in areas of intratumor necrosis. Work done on carcinoma of the thyroid revealed the presence of macrophages and CD45RO$^+$ T cells[4] and in carcinoma of the colon the presence of T cells again carrying the $\alpha\beta$ receptor.[5]

The role of the leukocyte infiltrate in carcinomas remains unclear. When activated, both tumor-associated macrophages and tumor-infiltrating lymphocytes are capable of destroying tumor cells.[26,27] However, using a reverse transcriptase polymerase chain reaction screen, we have shown that macrophage- and lymphocyte-activating cytokines such as interferon-γ and interleukin-2 are generally not expressed by ovarian tumors.[28] The apparent lack of CD4$^+$ cells would also imply that the infiltrate is immunologically inert. It is still not known whether macrophages associated with areas of microscopic necrosis are part of a host response to the tumor, causing local destruction, or whether they are simply there to remove necrotic debris from some other cause, such as the death of a blood vessel. The ratio between macrophages and tumor cells appears to be important; tumor-associated macrophages isolated from chemically induced tumors in mice promote tumor cell proliferation *in vitro* at ratios of <2:1 but exhibited nonspecific cytolytic activity at ratios of >20:1 without any specific activation.[12]

Although the role of the infiltrate is not understood, chemokines may account for its presence. In this paper, we have demonstrated a direct association between the number of chemokine-expressing cells and the leukocyte infiltrate. MCP-1 is a potent monocyte chemoattractant and has greater *in vitro* activity on these cells than other MCPs, MIP-1α, MIP-1β, or RANTES.[29] It is also active on T cells.[30,31] Some work suggests that MIP-1α is specific for CD8$^+$ T cells and MIP-1β for CD4$^+$ cells,[32,33] although other reports indicate that this relationship may not be absolute.[30] CD45RO$^+$ cells seem to possess greater migratory activity than CD45RA$^+$ cells in response to chemokines and RANTES may be most active on CD4$^+$, CD45RO$^+$ T cells[34] *in vitro*. Using *in situ* hybridization, we have shown that of the four C-C chemokines studied, the most frequently expressed were MCP-1 and MIP-1α. MCP-1 was more highly expressed by tumor cell islands than other chemokines. Although there was no significant statistical difference between the number of MCP-1-expressing cells in tumor and stroma after the Bonferroni correction was applied, MCP-1 expression by tumor cells is supported by our previous *in situ* observations and the fact that tumor cells isolated from ascites produced significantly more MCP-1 protein than macrophages.[15] In the stroma, MIP-1α-expressing cells were present in equivalent numbers to

those expressing MCP-1 and were more abundant than either MIP-1β or RANTES. The source of MIP-1α, MIP-1β, and RANTES was not clear, but these chemokines can all be derived from lymphocytes, and in particular, MIP-1α can be strongly expressed by $CD8^+$ T cells.[35] This is supported by the observation that the *in situ* signal for these chemokines was usually associated with nuclei that were smaller and more intensely staining than typical tumor cell nuclei within the tumors. Furthermore, whereas reverse transcriptase polymerase chain reaction revealed the presence of mRNA for all of these chemokines in tumor specimens, they were expressed by few ovarian tumor cell lines.[28]

To assess the relationship between the number of infiltrating cells and the number of chemokine-expressing cells, the total number of $CD68^+$ macrophages and $CD8^+$ T cells counted in frozen sections was correlated with the number of cells expressing each of the four chemokines. No correlation was found between MIP-1α and either of these two cell types, but the total number $CD8^+$ T cells correlated significantly with the number of cells expressing MCP-1 and RANTES. *In vitro*, MCP-1 has been shown to be chemoattractant for T cells and RANTES for $CD45RO^+$ T cells. Although these chemokines are also active on $CD4^+$ cells *in vitro*, the context in which they act *in vivo* may be very different. Furthermore, RANTES was expressed by a relatively low number of cells. Although the correlation between MCP-1 and the $CD68^+$ population was not statistically significant ($r_s = 0.5$; $P = 0.026$), the Bonferroni method used in this study is highly conservative and a weak correlation may exist. Other factors, particularly M-CSF, which is both a proliferative[36] and chemoattractant[37] factor for monocytes, may contribute to the macrophage population. Resident tissue macrophages can proliferate in response to M-CSF,[38] and double staining in our sections for CD68 and the proliferation marker MIB 1 revealed some cells positive for both (data not shown). In both adenocarcinoma of the breast[39] and ovary,[40,41] M-CSF is expressed by epithelial tumor cells, and its receptor, c-*fms*, is found on the surface of infiltrating macrophages. A positive correlation was also found between the number of $CD68^+$ macrophages and the number of $M\text{-}CSF^+$ tumor cells in adenocarcinoma of the breast.[39]

We have found that macrophages and $CD8^+$, $CD45RO^+$ T cells form the bulk of the infiltrating cell population, within both the tumor and stromal compartments. There are significantly more cells within the stroma than the tumor compartment, but only macrophages are found in high numbers within areas of necrosis. Of the four chemokines analyzed, MCP-1 and MIP-1α predominate. MCP-1 is expressed by significantly more tumor cells than any other chemokine, whereas MIP-1α is expressed by more stromal than tumor cells. MCP-1 may promote both the lymphocyte and macrophage infiltrate and MIP-1α may be expressed by $CD8^+$ T cells. We shall go on to examine the factors that may be responsible for promoting chemokine expression within the ovarian tumors, particularly the relationship between tumor cells and individual leukocyte subtypes. As tumor necrosis factor-α is known to be expressed by both tumor cells and tumor-associated macrophages in this disease[42] and can stimulate MCP-1 expression in a variety of cell types, this cytokine provides one possible link between the infiltrating cell population and the pattern of expression of chemokines observed within these tumors.

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