NKR-P1+ Cells Localize Selectively in Rat 9L Gliosarcomas but Have Reduced Cytolytic Function

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ABSTRACT

To better understand immune responses to brain tumors and to develop possible approaches for immunotherapy, we have investigated the leukocyte populations infiltrating the rat 9L gliosarcoma. By immunocytochemical analyses of the cells infiltrating the tumor, we observed a substantial number of cells expressing natural killer cell receptor protein 1 (NKR-P1), a marker expressed only on rat natural killer cells capable of non-MHC-restricted cytotoxicity. Previous investigations have determined the existence of three populations of NKR-P1+ lymphocytes in normal rats, including NKR-P1high/T-cell receptor (TCR)/CD3-CD5- (5-15%), NKR-P1dim/TCRa/CD3+/CD5+ (5-15%), and NKR-P1low/TCRγδ/CD3+/CD5+ (0-5-2%). By one-parameter flow cytometry, it was determined that NKR-P1+ cells constituted 30-60% of the lymphocytes in 9L tumors. Among splenic lymphocytes or peripheral blood leukocytes, NKR-P1high cells are 1.5-4.5 times more numerous than NKR-P1dim cells. In striking contrast, NKR-P1dim cells were 4-5 times more numerous than NKR-P1high cells among lymphocytes isolated from 9L tumors. Using quantitative analyses of laser confocal microscopic scans, we determined that NKR-P1dim cells were ~4 times as numerous as NKR-P1high cells in situ, confirming flow cytometric findings. By two-color flow cytometric analyses, it was observed that ~5-10% of the cells were NKR-P1high/CD5+/TCR-, a phenotype representative of NK cells. Also, ~11-25% of the cells were NKR-P1dim/CD5+/TCR- cells, corresponding to the T-cell subset with non-MHC-restricted lytic function. In addition, we observed a cell population among 9L-derived lymphocytes with a NKR-P1dim/CD5+/TCR- phenotype (~15-25%). Cells of this phenotype have not been reported previously, and most likely represent NK cells down-modulated for expression of NKR-P1. Alternatively, they might represent cells of unknown origin or cells down-modulated for expression of T-cell markers in the microenvironment of 9L tumors. We also compared the lytic capacity of NKR-P1+ populations derived from normal animals and from 9L gliosarcomas. In these experiments, it was determined that although cells isolated from 9L tumors had some capacity to lyse tumor target cells, they were clearly less efficient than cells isolated from normal splenocytes. Cumulatively, these data suggest that there is selective localization of cells capable of mediating antitumor responses in 9L, but that tumor-associated factors may down-regulate their function and expression of NKR-P1.

INTRODUCTION

Primary malignancies of the central nervous system cause the deaths of more than 12,000 patients annually (1). Malignant gliomas make up the majority of these tumors, and they are the most frequent solid tumor of childhood cancers (1, 2). Recent reports also suggest that these tumors are becoming increasingly prevalent among the elderly (1, 2). Despite important advances in neuroimaging and neurosurgical technique, treatment of most central nervous system tumors has been ineffective, and the prognosis for these patients remains poor (1, 3, 4). Cytoreductive surgical procedures are of limited benefit for malignant gliomas, because they infiltrate adjacent normal tissues, and resection with a generous margin is not possible in the brain, as opposed to solid tumors in other sites. Similar concerns for the preservation of normal nervous tissues limit the usefulness of radiation therapy and chemotherapy for treatment of gliomas, and a balance between efficacious treatment and deleterious effects on brain function has not been achieved (1, 5). Recurrence is generally within months and in some disease entities (e.g., glioblastoma multiforme), up to 80% of patients die within a year (1). Although some adjunctive treatments such as interstitial brachytherapy have shown promise, the prognosis for patients with primary brain tumors has not improved significantly (6-11).

Some aspects of the biology of gliomas have suggested that immunological approaches to therapy may be of benefit. For instance, there is a lymphoid/myeloid infiltrate in 40-80% of both well- and poorly differentiated gliomas, suggesting an immune response to the tumor (reviewed in Ref. 12). A MN3 infiltrate can be demonstrated in perivascular regions within and adjacent to Virchow-Robin spaces (13-15) and in the tumor tissue and parenchyma of the adjacent brain tissue (16, 17). This cellular infiltrate has some characteristics similar to those found in extraneural solid tumors (18-21). Phenotypic analyses have suggested that the cellular infiltrate consists predominantly of T cells (mostly CTL/suppressor cells; Refs. 17, 22, and 23), some B helper cells (17), and macrophages (24, 25). However, very few B cells were present, at least in anaplastic gliomas (26). Information about possible NK cell infiltration of human gliomas is limited, as commonly used markers such as CD56 (N-CAM) and CD57 (HNK-1) are also expressed on neuronal tissues (27, 28). However, one report suggests that as many as 40% of the infiltrating cells bear NK cell lineage markers (29). These data indicate that there is a frequent lymphoid infiltration into gliomas, but the phenotypic and functional characterization of the infiltrating cells remains incomplete. Because glioblastomas grow progressively and invasively, it is clear that, under most circumstances, the tumor-infiltrating lymphocytes do not exert potent antitumor functions. Although the use of activated lymphocytes for adoptive cellular immunotherapy has generally been ineffective as therapy for glioblastoma multiforme, sustained clinical responses were seen in a small proportion of patients (30). Given the recent interest in the use of BRMs to activate lymphocytes in situ, it may be possible to shift the balance toward effective host resistance and control of tumor growth. To explore this possibility, it is essential to determine which cells of the immune system have the greatest potential for localization in the tumors, what regulates their localization, and potentially what regulates their state of activation, both positively and negatively.

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While an extensive evaluation of lymphoid infiltration of preclinical brain tumor models is also lacking, several reports have indicated that there is a pronounced infiltration of lymphoid cells into 9L tumors (31) in rats. For instance, Wen et al. (32, 33) reported that there were as many as 40% CD45+ cells in the tumor mass, including up to 20% CD4+ cells, up to 12% CD8+ cells, but less than 2% of cells with a B-cell phenotype (i.e., OX33+). There were many class II+ (OX6+) cells along the needle track (for implantation) and in the periphery of the tumor. Although there were apparently MHC class II+ lymphoid cells present, the majority of these cells were reported to have a morphology consistent with that of microglial cells (32, 33). In another report from the same group, it was demonstrated that CD45+ cells were distributed mostly in the periphery of lacZ-transfected 9L tumors but were not always adjacent to tumor cells (34). Interestingly, the improved capacity to identify tumor cells, conferred by the lacZ transfection, allowed a determination that the lymphoid cells, located primarily in the periphery of the central tumor mass, were also associated with the infiltrative tumor margins (34).

In a similar model using chemically induced, immunogenic F98 and D74 gliomas, it was found that nonimmune rats had a cellular infiltrate consisting of ~20% CD8+ cells, ~20% CD4+ cells, and ~6% asialoGM1+ cells (35). Interestingly, in experiments comparing rats immunized with F98 or D74 and nonimmune rats, it was determined that there was a marked increase in CD8+ cells in the tumors of immune rats (35). Although 9L has been shown to be immunogenic (36), a comparison of the phenotypes of infiltrating cells in 9L tumors in immune and nonimmune rats has not been reported.

It is clear that 9L tumors, and others as well, have a substantial lymphoid infiltrate and yet grow progressively and rapidly. However, there is insufficient precise information about the phenotype of the infiltrating cells and very little data on the functional status of those cells. For instance, data reported to date on cytolytic effector cells have only indicated infiltration of CD8+ cells into 9L. In the rat, CTLs and >80% of NK cells express CD8α (37, 38), and therefore, the sole use of this marker is less than satisfactory for evaluation of infiltration of either T cells or NK cells into the tumor. Similar concerns about human studies must also be raised, as 20–50% of human NK cells express CD8α homodimers (39). There are no reports of cells expressing pan-T markers such as CD2, CD3, CD5, or TCR, which, coupled with numbers of CD4+ and CD8+ infiltrating cells, would give an accurate reflection of the T-cell compartment in 9L tumors. As far as rat NK cells are concerned, the use of anti-NKR-P1, coupled with anti-CD3 or anti-TCR, would give an accurate reflection of the numbers of NK cells (i.e., with the phenotype of NKR-P1high TCRαβ+/CD3+) infiltrating 9L tumors (40–42). Because the human homologue of NKR-P1 has been described recently (43), similar experiments can also be performed in human tumors. Subpopulations among T cells and among NK cells can be identified phenotypically and functionally and according to their capacity to respond to cytokines (reviewed in Ref. 44). On the basis of these facts, it would be of considerable interest to determine whether the tumor is selectively infiltrated by subsets of T or NK cells that might be hypofunctional or inactivated in the brain tumor microenvironment but which might have relevant functional capabilities or have the potential to be selectively activated or up-regulated by various BRMs.

There are numerous reports suggesting a reduction in immune function in tumor-bearing individuals, including those with brain tumors (reviewed in Ref. 45). Similar data have been reported in regard to NK cell function in the spleens of 9L tumor-bearing rats (46, 47). However, in the case of the rat model, the data reflect NK activity among splenocytes without a determination of the percentage of NK cells present in either normal or tumor-bearing rats. These data might be due either to a reduction in number of NK cells in tumor-bearing rats based on their migration out of the spleen, or to a reduction in NK activity on a per-cell basis. To distinguish between these two quite divergent possibilities, a more thorough evaluation, comparing lytic activity of equal numbers of NK cells sorted from spleen of normal and tumor-bearing rats, is warranted. Regarding the functional status of NK or T cells among 9L infiltrating lymphocytes, it has been reported that there is appreciable specific CTL activity among tumor-derived lymphocytes in immunized rats (35). NK activity among 9L tumor-derived lymphocytes was weak but detectable in the immunized rats. However, this function of 9L tumor-derived lymphocytes was not compared between immunized and nonimmunized rats (35). On the basis of current information, it is possible to conclude that, although there may be some suppression of immunity, cells expressing cytolytic activity can be isolated from among 9L tumor-infiltrating cells after immunization.

There is also limited information available on the status of activation of cells infiltrating the tumor or their response to in vivo treatment with BRMs. As mentioned above, the available data on MHC class II+ cells do not clearly define the state of activation of lymphocytes in the tumor (32, 33). There is, however, a report on expression of class II on infiltrating cells after in vivo IFN treatment of 9L tumor-bearing rats. In this case, there was an apparent increase in inflammation in the tumor, and there was an increase in MHC class II+ MNCs in the periphery. However, there was not an extensive characterization of the phenotype of the infiltrating cells (33). Use of markers in the rat such as anti-CD25 (activated T and NK cells; Ref. 38), anti-class II (on defined populations of lymphoid and myeloid cells; Ref. 38), or anti-gp42 (IL-2-activated NK cells; Ref. 48) would provide some indication as to the level of activation of the cells. Information of this nature will be valuable in directing the use of BRMs and in tailoring their use for the greatest therapeutic efficacy.

The data generated to date provide some useful information on lymphoid infiltration of the 9L gliosarcoma. However, the level of understanding of immune responses in this model would be improved substantially by the use of a greater number of lymphoid-specific markers in two- and three-color flow cytometric and/or confocal microscopic studies. In this report, we present data on the selective nature of the cellular infiltrate in 9L gliosarcomas and on the functional status of those cells.

### MATERIALS AND METHODS

**Chemicals and Reagents.** Tissue culture medium (RPMI 1640), HBSS, FBS, l-glutamine, and Pen/Strep were purchased from Life Technologies, Inc. (Grand Island, NY). mAbs, unconjugated or conjugated with FITC, recognizing rat CD3 (I-F4; pan-T-cell marker), CD4 (W3/25; T-helper cell marker), CD5 (OX19; pan-T-cell marker), CD8ε (OX8; CTL and NK cell marker), CD25 (OX39; activated T cells), CD45 (OX1; hematopoietic cells), TCRβ (R73.1), TCRγδ (V65) and class II (OX6; macrophages, B cells, subsets of activated NK and T cells) were purchased from PharMingen, Inc. (San Diego, CA). Cy3-conjugated goat antimouse immunoglobulin was purchased from Accurate Chemical and Scientific Co. (Westbury, NY). Biotin-, FITC- and Cy5-conjugated anti-NKR-P1 (mAb 3.2.3) was prepared as described (40). Horseradish peroxidase-conjugated streptavidin was purchased from PharMingen, Inc.

**9L Tumor Model.** 9L tumors were established intracranially by stereotactic implantation of 5 × 10⁶ cells per rat, as described previously (32–34). In some instances, 9L tumors were also established by s.c. injection of 2 × 10⁶ cells/site (two sites/rat) over the abdomen. Tumors were disaggregated mechanically using the end of a syringe plunger in a Petri dish containing RPMI 1640 supplemented with 5% FBS.

**Cell Culture.** YAC-1 tumor target cells were maintained as stationary suspension cultures in RPMI 1640 supplemented with 5% FBS, pen-strep, and l-glutamine (CM) in a humidified, 37°C incubator containing 5% CO₂ in air. Cultures were split every other day to maintain cells in a log phase of growth.

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9L tumor cells were maintained as stationary adherent cultures in CM under the same conditions and were split at or immediately prior to confluency of flasks. 9L cells were treated with trypsin-versene for removal from flasks and were then cultured a minimum of 4 h at 37°C before label with chromium and use in cytotoxicity assays. Sorted populations of NKR-P1 bright and NKR-P1 dim cells were cultured in CM with the addition of recombinant human IL2 at 500-1000 U/ml as described previously (40—42).

Flow Cytometric Analyses and Cell Sorting. One-, two-, and three-color flow cytometric analyses and sorting experiments were performed as described previously (41) using a Becton Dickinson FACStar Plus cytometer. In all experiments, the population of cells within a lymphoid gate was determined by forward versus orthogonal light scatter, and isotype-matched control mAbs were used for setting markers. Analyses of data were performed using ReProMan software (TrueFacts Software, Seattle, WA).

Immunocytochemistry. Immunocytochemical analysis of cellular infiltration of established 9L tumors was carried out on stained frozen sections as described previously (49).

Cytopsins. Cytopsins of sorted populations of cells were prepared as described previously using a Shandon Cytocentrifuge (Sewickley, PA; Ref. 41). Cells were stained using Wright's Giemsa stain as described (41). Two hundred cells/slide were tabulated.

Laser Confocal Microscopy. Laser confocal microscopic analyses were carried out in the Structural Biology and Imaging Center of the University of Pittsburgh on a Molecular Dynamics 2001 Confocal microscope (Sunnyvale, CA). Images were analyzed using ImageScape (Silicon Graphics, Sunnyvale, CA). 9L tumors were perfused with 1% paraformaldehyde for 1–3 h before immersion in 30% sucrose and freezing at —70°C. Sectioning of tumors was performed on a cryostat at a thickness of 6–8 μm/slide.

Cytotoxicity Assays. Cytolytic function of sorted populations of cells was tested by either a 4- or 18-h microcytotoxicity assay as described previously (40—42). Cells isolated from spleens of normal rats and cells isolated from established 9L tumors (intracranial and subcutaneous) were used as effector cell populations. 51Cr-labeled YAC-1 or 9L tumor cells were used as targets. Percentage of cytotoxicity was determined by the following formula:

\[
\text{% specific cytotoxicity} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total releasable} - \text{spontaneous release}} \times 100
\]

Comparisons of efficiency of cytolytic function between cell populations were assessed by calculation of LU3×10^6 cells as described (41).

RESULTS

Multiple Subsets of NKR-P1 \(^+ \) Cells Infiltrate 9L Gliosarcomas. Initially, we investigated the presence of NKR-P1 \(^+ \) cells localized in established 9L tumors using immunocytochemical staining of tumor sections with biotinylated anti-NKR-P1. As illustrated in Fig. 1, NKR-P1 \(^+ \) cells were associated with 9L tumors but were not found in normal brain. We have previously defined three subsets of NKR-P1 \(^+ \) lymphocytes in rats, including NKR-P1 bright/TCR \(^+ \)/CD3 \(^- \)/CD5 \(^+ \) (i.e., NK cells; Refs. 40—42, 50), NKR-P1 dim/TCR\(\alpha\beta^+ \)/CD3 \(^+ \)/CD5 \(^+ \) (i.e., \(\alpha\beta\) T cells; Refs. 40—42, 50), and NKR-P1 dim/TCR\(\gamma\delta^+ \)/CD3 \(^+ \)/CD5 \(^+ \) (i.e., \(\gamma\delta\) T cells). As immunocytochemical analyses are not capable of defining the relative level of expression of a given epitope in sections, we carried out one-parameter flow cytometric analyses of expression of NKR-P1 on lymphoid cells isolated from disaggregated 9L tumors. Data in Fig. 2 demonstrate that NKR-P1 \(^+ \) cells of both NKR-P1 \(\text{dim} \) and NKR-P1 \(\text{bright} \) phenotypes were present, and that the majority of NKR-P1 \(^+ \) cells had the NKR-P1 \(\text{dim} \) phenotype. Data in Table 1, summarizing the results from three representative preparations of lymphoid cells from 9L tumors, indicate that NKR-P1 \(^+ \) cells were a significant percentage, if not the majority, of total lymphoid cells from 9L tumors, and that NKR-P1 \(\text{dim} \) cells were present in approximately 4–5 times greater numbers than NKR-P1 \(\text{bright} \) cells (41 ± 7.0% versus 8 ± 1.7%, respectively). These data are in marked contrast to the percentages of NKR-P1 \(^+ \) cells found in spleen and blood of rats, where NKR-P1 \(^+ \) cells constitute ~5–15% of total lymphocytes, and NKR-P1 \(\text{bright} \) cells are present in 1.5- to 4.5-fold greater numbers (40—42, 49). As the flow cytometric analyses were performed on cells disaggregated from 9L tumors without treatment with proteolytic enzymes, we considered the possibility that the relatively high proportion of NKR-P1 \(\text{dim} \) cells might be due to a technical artifact, with NKR-P1 \(\text{bright} \) cells binding more efficiently to tumor cells and thereby being lost in the isolation of single-cell suspensions by density-gradient centrifugation during which aggregated/conjugated cells could be lost. Therefore, we sought to determine the relative number of NKR-P1 \(\text{bright} \) versus NKR-P1 \(\text{dim} \) cells in situ using confocal microscopic analyses. As illustrated in Fig. 2.
found to contain ~6% NKR-P1^{bright}/CD5^- (Fig. 4C, Quadrant 1), ~22% NKR-P1^{dim}/CD5^- (Fig. 4C, Quadrant 1) and ~22% NKR-P1^{dim}/CD5^+ (Fig. 4C, Quadrant 2) cells. Data using anti-NKR-P1 versus anti-TCRαβ (Fig. 4D) indicated that there were ~6% NKR-P1^{bright}/TCRαβ^- (Fig. 4D, Quadrant 1), ~22% NKR-P1^{dim}/TCRαβ^-, and ~22% NKR-P1^{dim}/TCRαβ^+ (Fig. 4D, Quadrant 2) cells. These data are similar to the one-parameter flow experiments in that there are significantly more NKR-P1^{dim} than NKR-P1^{bright} cells among tumor-associated lymphocytes. However, in addition, the two-parameter flow cytometric analyses revealed additional differences between normal splenic and tumor-derived lymphocytes. In particular, there was a large population (~22% in this experiment) of NKR-P1^{dim}/CD5^- cells among tumor-derived cells (Fig. 4C, Quadrant 1), whereas this population was not observed among lymphocytes from the spleen of normal rats (Fig. 4B, Quadrant 1). Similarly, a large population of NKR-P1^{dim}/TCRαβ^- cells (~22%; Fig. 4D, Quadrant 1) was seen among tumor-derived cells. A similar population of cells could be identified among splenocytes of normal rats (~2%; Fig. 4B, Quadrant 1), and in normal rats these have been determined to be NKR-P1^{dim}/TCRγδ^- cells.

To investigate the possibility that this large 9L tumor-associated population of NKR-P1^{dim}/TCRαβ^- cells might also be TCRγδ^- cells, we carried out flow cytometric analyses using anti-NKR-P1 versus anti-TCRαβ. In several experiments, we observed that there were very few TCRγδ^- cells among tumor-derived lymphocytes (3% ± 1.9%, n = 4; data not shown), and this small population of cells could not account for the much higher proportion of NKR-P1^{dim}/TCRαβ^- cells. Furthermore, the NKR-P1^{dim}/TCRγδ^- cells have been found to express CD5, whereas 9L-derived NKR-P1^{dim} cells lacked CD5 (Fig. 4C, Quadrant 1). Using two- and three-color flow cytometric analyses, we also determined that the NKR-P1^{dim}/CD5^-/TCRαβ^- cells expressed CD3, whereas NKR-P1^{dim}/CD5^+/TCRαβ^- cells lacked expression of CD3 (data not shown). As would be expected in these experiments, the NKR-P1^{bright}/CD5^-/TCR^- cells also lacked expression of CD3 (data not shown). We considered the possibility that the NKR-P1^{dim}/CD5^-/TCR^- cells were not of hematopoietic origin and were in fact 9L cells that expressed low levels of NKR-P1, and we therefore carried out three-color flow cytometric analyses using anti-NKR-P1, anti-CD5, and anti-CD45. In these experiments, it was determined that all NKR-P1^+ populations expressed CD45 (data not shown), indicating their hematopoietic origin and ruling out the possibility that NKR-P1^+ 9L cells contributed to the results.

In rats, it has been reported that NKR-P1 is expressed at high levels on NK cells, at low levels on two small subsets of T cells, and at very low levels on neutrophils (40–42, 50–52). Because the apparently unique population of cells with the NKR-P1^{dim}/CD5^-/TCR^- phenotype was contained in the lymphoid gate and was found among MnIs isolated on Ficol, it seemed most likely that they were MnIs and not a contaminating population of neutrophils. However, to demonstrate directly that the NKR-P1^{dim}/CD5^-/TCR^- cells were MnIs, we sorted populations of NKR-P1^{bright}/CD3^-, NKR-P1^{dim}/CD3^+, and NKR-

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| Mean ± SD | 49 ± 8.7 | 41 ± 7.0 | 8 ± 1.7 |

a Percentage positive staining with mAb 3.2.3, compared to isotype control staining using cells derived from day 14 9L tumors implanted intracranially.
P1dim/CD3+ cells and prepared cytospins for morphological analysis of each population. As illustrated in Fig. 5, most of the NKR-P1dim/CD3− (Fig. 5, upper left) and NKR-P1dim/CD3+ (Fig. 5, upper right) cells were agranular lymphoid cells. There was, however, a small percentage of neutrophils (≤5%) in the NKR-P1dim/CD3− population. The NKR-P1bright/CD3− (Fig. 5, lower left) cells had the characteristic morphology of LGL populations of NK cells. For size comparisons, a cytospin of in vitro-cultured 9L cells was also prepared, and the 9L cells are substantially larger than either of the three lymphoid compartments. On the basis of the size differential between the NKR-P1+ lymphoid cells and 9L, it is unlikely than any 9L cells would be included within the lymphoid gate used for analysis of phenotype in our experiments. However, it must be noted that, in this figure, the 9L cells were trypsinized for removal from flasks and therefore have a round morphology, whereas they generally have a fibroblast-like appearance in culture.
Two sets of cytospins were analyzed for the presence of MNs and for the percentage of the MN cells with LGL or non-LGL morphology (Table 2). These data demonstrate that the cells of unique phenotype (i.e., NKR-P1dim/CD3dim/CD5+/TCRαβ−) are agranular MNs in nature and do not represent a contaminating population of neutrophils.

To characterize further the state of responsiveness, we also investigated whether the cells infiltrating 9L tumors expressed leukocyte activation markers. We determined that very few cells (~10%) expressed the α chain of the high-affinity receptor for IL2 (Tac, p55, CD25) (Fig. 6D, Quadrants 2 and 4). Virtually none of the NKR-P1dim cells were found to express CD25. This is comparable to the percentage of cells expressing this marker among normal splenic lymphocytes and peripheral blood leukocytes. Because it has been reported that a significant percentage of cells in sections of 9L tumor, or along the needle track after implantation of 9L, expressed class II (OX6, Ia-like antigen; Refs. 32 and 33), we also determined by flow cytometric analyses what percentage of cells expressed class II. Because class II expression on NK cells is indicative of their activation (38), we also determined whether NKR-P1dim cells in 9L expressed class II. Data in Fig. 6 demonstrate that ~40% of cells expressed class II (Fig. 6C, Quadrants 2 and 4). The percentage of OX6+ cells among NKR-P1dim, NKR-P1bright, and NKR-P1dim cells, in three representative experiments, is presented in Table 3. It was determined that <1% of NKR-P1bright cells expressed class II, indicating that none of the typical NK cells were present in an activated state. In all experiments, a substantial proportion of the NKR-P1dim cells were found to express class II (mean of 14 ± 5.4% of the cells, and ~50% of NKR-P1dim cells). However, it was not known whether expression of class II is indicative of activation for NKR-P1dim cells.

Taken together, these data indicate clearly that there is a greater percentage of NKR-P1dim cells localizing in the 9L tumors than in...
spleen or peripheral blood leukocytes. Furthermore, a substantial proportion of tumor-derived lymphocytes have a unique phenotype (i.e., NKR-P1\(^{\text{dim}}\)/CD3\(^+\)/CD5\(^+\)/TCR\(^+\)).

**Evaluation of the Functional Status of NKR-P1\(^+\) Cells Derived from Established 9L Gliosarcomas.** NKR-P1\(^+\) cells represent a substantial proportion of the lymphoid cells infiltrating 9L gliosarcomas, and it is known that antitumor lytic function corresponds with expression of NKR-P1 in uncultured lymphoid cells (40–42, 50). However, 9L tumors display progressive growth and lethality leading to death of implanted rats (31–35). Therefore, one might hypothesize that the NKR-P1\(^+\) cells in 9L tumors are not capable of antitumor activity because of either (a) an inability to recognize 9L tumors, (b) the lack of suitable antitumor effector function; or (c) suppression of their antitumor function by the tumor microenvironment. To distinguish among these possibilities, we tested the antitumor lytic activity of NKR-P1\(^+\) cells infiltrating 9L. As illustrated in Fig. 7A, sorted NKR-P1\(^{\text{bright}}\) cells (i.e., NK cells) were able to lyse YAC-1 target cells. NKR-P1\(^{\text{dim}}\) cells had some level of lytic function against YAC-1, but were much less efficient than the NKR-P1\(^{\text{bright}}\) cells. In contrast, freshly isolated NKR-P1\(^{+}\) cells derived from 9L tumors had no lytic activity against 9L (data not shown). However, we sorted NKR-P1\(^{\text{bright}}\) cells derived from lymphocytes infiltrating 9L tumors, cultured them for 5 days in 500 U/ml recombinant IL2, and assessed their capacity to lyse YAC-1 and 9L target cells. Data in Fig. 7B indicate that IL2-activated, 9L tumor-derived NKR-P1\(^{\text{bright}}\) cells were capable of lysing both YAC-1 and 9L target cells.

There are numerous reports of immunosuppression of NK activity among cells derived from tumors and from the periphery of tumor-bearing individuals (reviewed in Ref. 44). There have also been reports of suppression of NK activity among splenocytes of rats bearing 9L tumors (46, 47), but these did not assess relative percentages of NK cells among lymphocytes isolated from normal versus tumor-bearing rats. Thus, it was not possible to determine whether the reduced NK activity in 9L tumor-bearing rats was a reflection of a lesser percentage of NK cells or reduced lytic function on a per-cell basis. Therefore, we directly compared the lytic capacity of NKR-P1\(^+\) cells derived from the spleen of normal rats and from established 9L tumors. The 9L-derived NK (i.e., NKR-P1\(^{\text{bright}}\)) cells were found to be less lytically active than NK cells from normal rats (Table 4). LUs were calculated for the NKR-P1\(^{\text{bright}}\) cells sorted from normal splenocytes and from 9L, and these populations were found to have 206 and 32 LU\(\text{yAC-1}\)/10\(^6\) cells, respectively. Comparing lytic activity of NKR-P1\(^{\text{bright}}\) versus NKR-P1\(^{\text{dim}}\) cells again confirmed that the NKR-P1\(^{\text{bright}}\) cells have a much greater capacity to lyse YAC-1 target cells than NKR-P1\(^{\text{dim}}\) cells (206 versus 12 LU\(\text{yAC-1}\)/10\(^6\) cells). Comparison with Student’s t test indicated that there were statistically significant differences (P \(\leq 0.05\)) in cytotoxicity mediated by NKR-P1\(^{\text{bright}}\) cells from spleen and 9L at 50:1 in experiment 1 and at 10:1, 5:1, and 2.5:1 in experiment 2. While the level of activity was low, there was an apparently greater ability of NKR-P1\(^{\text{dim}}\) cells from normal rats to lyse YAC-1 target cells than NKR-P1\(^{\text{dim}}\) cells sorted from 9L-infiltrating lymphoid cells (12 versus 8 LU\(\text{yAC-1}\)/10\(^6\) cells). However, these differences were not statistically significant. These data indicate clearly that 9L tumor-derived NKR-P1\(^+\) cells are less lytically active on a per-cell basis than cells derived from normal animals.

**DISCUSSION**

Data from numerous investigations indicate that a large proportion of gliomas have a leukocyte infiltrate (12–25, 31–37). However, there has not been an extensive characterization of the phenotype and function of these infiltrating cells. In view of the potential use of immunotherapy for the treatment of brain tumors, we have sought to develop a preclinical model in rats and to investigate the lineage relationships and function of leukocytes infiltrating the tumor. In that regard, we have determined that there is a substantial infiltration of

**Table 2** Morphological analyses of NKR-P1\(^{\text{bright}}\)/CD3\(^+\), NKR-P1\(^{\text{dim}}\)/CD3\(^+\), and NKR-P1\(^{\text{dim}}\)/CD3\(^+\) cells among 9L-infiltrating leukocytes

<table>
<thead>
<tr>
<th>% MN</th>
<th>% LGL</th>
<th>% nLGL</th>
<th>% MN</th>
<th>% LGL</th>
<th>% nLGL</th>
<th>% MN</th>
<th>% LGL</th>
<th>% nLGL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>95</td>
<td>0</td>
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<tr>
<td>2</td>
<td>100</td>
<td>88</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>94</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>97</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Fig. 6.** Expression of activation markers on 9L-infiltrating lymphocytes. Lymphocytes from disaggregated 9L tumors (intracranial) were incubated with Cy5-conjugated (FL4) anti-NKR-P1 (mAb 3.2.3) and FITC-conjugated (FL1) anti-CD5 (OX19) or anti-class II (OX6) or anti-CD25 (OX39). Staining was based on isotype control staining. A. isotype-matched control staining; B. NKR-P1 versus CD5; C. NKR-P1 versus OX6; D. NKR-P1 versus CD25. The percentages of positive-staining cells in the lymphoid gate were as follows. A. Quadrant 1, 0.4; Quadrant 2, 19.7; Quadrant 3, 40.6; Quadrant 4, 29.3. B, Quadrant 1, 19.7; Quadrant 2, 17.1; Quadrant 3, 46.8; Quadrant 4, 22.7. C, Quadrant 1, 35.8; Quadrant 2, 1.8; Quadrant 3, 54.7; and Quadrant 4, 7.7.
lymphoid cells into 9L gliosarcomas, and that there is an apparent predilection for NKR-P1+ cells to localize or be retained in the tumor. Among lymphoid cells in the spleen and peripheral blood of normal rats, NKR-P1+ cells constitute ~5–15% of total cells. Among NKR-P1+ lymphocytes, there are generally 1.5–4.5 times as many NKR-P1bright cells as NKR-P1dim cells (40–42). In contrast, NKR-P1+ cells isolated from 9L gliosarcomas constituted ~30–60% of the total, and NKR-P1dim cells were generally present in ~4–5 times greater numbers than NKR-P1bright cells. Most interesting among these data was the identification of a substantial proportion of 9L-infiltrating lymphoid cells, which were characterized phenotypically as NKR-P1dim/CD3+/CD5−/TCR−/CD25+/CD45+. These cells were also characterized morphologically as agranular MNs. By light scatter, they were associated with the lymphoid population. These data all suggest that these cells are lymphocytes of unknown origin. Several hypotheses could be forwarded for the origin of these cells. (a) This compartment of cells might represent a population of large agranular lymphocytes known to be precursors to NK cells (53). Although this is possible, our preliminary results suggest that culture of sorted NKR-P1dim/CD3− cells in IL2 did not induce a phenotypic change to NKR-P1bright cells (data not shown). However, culture with this single cytokine may not be sufficient to support efficient differentiation of the precursors. (b) NKR-P1dim/CD3−/CD5−/TCR− cells might represent a population of NKR-P1bright NK cells that were induced to down-modulate expression of NKR-P1. Tumor-related factors might induce phenotypic changes in NK cells, as there are clearly tumor-associated factors that alter NK cell function (reviewed in Ref. 44). For example, TGF-β2 has been reported to be produced in significant amounts in gliomas (54–56), and this cytokine is a potent inhibitor of NK cells. We have observed that 9L gliosarcomas produce TGF-β2 and that supernatants of fresh-explants of 9L are suppressive of NK cell responses to IL2 (data not shown). Our observation of low cytolytic activity by 9L-derived NKR-P1bright cells supports further the possibility of down-regulation of NK function by 9L tumor-associated factors. We have also determined that recombinant TGF-β2 inhibits up-regulation of NKR-P1 induced by IL2 and IL15 (data not shown), suggesting an inverse relationship between the effects of TGF-β2 and expression of NKR-P1. These data lend strong support to this second hypothesis. (c) NKR-P1dim/CD3+/CD5−/TCR− cells might represent a compartment of NKR-P1dim T cells that have down-modulated expression of CD3, CD5, and TCR. However, we have examined the phenotype of NKR-P1dim T cells after culture in rTGF-β2 and have not seen an effect of this cytokine on expression of CD3, CD5, or TCRβ (data not shown). (d) These cells might represent a unique subset of cells not defined previously as yet another possibility. These cells might also be a heterogeneous population containing any or all of the hypothesized compartments, but we have not yet been able to demonstrate any heterogeneity in lymphocytes in this population. Although the investigation of the origin of NKR-P1dim/CD3+/CD5−/TCR− might provide clues to the regulation of localization and function of cells infiltrating gliomas, it is also of much interest that such a large proportion of cells infiltrating 9L are of the NKR-P1dim/CD3+/CD5−/TCR− phenotype. Among splenocytes, these cells may represent 1–5% of total lymphocytes, whereas among 9L-derived lymphocytes they may constitute as much as 15–25% of the total cells. The analogous population of cells in mice, i.e., NK1.1+/TCRβ+, are

### Table 3 Expression of OX6 (class II) on NKR-P1+ and NKR-P1− cells infiltrating 9L gliosarcomas

<table>
<thead>
<tr>
<th>Experiment</th>
<th>OX6+</th>
<th>NKR-P1dim</th>
<th>NKR-P1−</th>
<th>NKR-P1bright</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40%</td>
<td>17%</td>
<td>23%</td>
<td>≤1%</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>16%</td>
<td>28%</td>
<td>≤1%</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>6%</td>
<td>13%</td>
<td>≤1%</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>35 ± 11.6</td>
<td>14 ± 5.4</td>
<td>21 ± 6.2</td>
<td></td>
</tr>
</tbody>
</table>

*a* Percentage positive staining using cells derived from day 14 9L tumors implanted intracranially.

### Table 4 Comparison of lytic activity against YAC-1 target cells of NKR-P1+ cells isolated from normal splenocytes versus established (day 14) 9L gliosarcomas

<table>
<thead>
<tr>
<th>NKR-P1bright</th>
<th>Normal spleen</th>
<th>9L-derived*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal spleen</td>
<td>6 ± 0.8</td>
<td>1 ± 3.0</td>
</tr>
<tr>
<td>9L-derived</td>
<td>5 ± 0.6</td>
<td>1 ± 0.5</td>
</tr>
<tr>
<td>10:1</td>
<td>3 ± 2.9</td>
<td>3 ± 1.3</td>
</tr>
<tr>
<td>25:1</td>
<td>5 ± 0.7</td>
<td>3 ± 1.5</td>
</tr>
<tr>
<td>50:1</td>
<td>12.25:1</td>
<td>33 ± 5.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NKR-P1dim</th>
<th>Normal spleen</th>
<th>9L-derived</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal spleen</td>
<td>30 ± 3.8</td>
<td>22 ± 6.9</td>
</tr>
<tr>
<td>9L-derived</td>
<td>21 ± 2.4</td>
<td>17 ± 2.8</td>
</tr>
<tr>
<td>5:1</td>
<td>29 ± 5.2</td>
<td>14 ± 0.6</td>
</tr>
<tr>
<td>2.5:1</td>
<td>24 ± 3.0</td>
<td>11 ± 6.5</td>
</tr>
<tr>
<td>LL-610*</td>
<td>32 ± 8</td>
<td>12 ± 12</td>
</tr>
</tbody>
</table>

*a* Cells sorted from day 14 9L tumors grown subcutaneously.

* E:T ratio.

* Percentage specific cytotoxicity ± SD against YAC-1 target cells in 18 hr 51Cr release microcytotoxicity assays.

* p < 0.05 when comparing splenic vs 9L-derived NKR-P1bright cells.

* ND, not determined.
potent producers of cytokines such as IL4 (57–60), and a role for this cytokine in antitumor responses or suppression of antitumor responses must be considered. In that regard, Bozik et al. (61) have determined that 9L gliosarcoma cells transfected with IL4 initially form tumors, but the tumors subsequently regress. These data suggest that an evaluation of the production of IL4, or reduced capacity for production of IL4, by NK-PI \textsuperscript{+}\textsuperscript{+}/CD3\textsuperscript{+}/CD7\textsuperscript{+} TCR\textsuperscript{+} cells infiltrating 9L will be of interest.

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