Cancer Immunol Immunother (1994) 38: 135–141

Cancer Immunology Immunotherapy

Transfection of the mouse ICAM-1 gene into murine neuroblastoma enhances susceptibility to lysis, reduces in vivo tumorigenicity and decreases ICAM-2-dependent killing

Emmanuel Katsanis[1], Maria A. Bausero[1], Hong Xu[2], Paul J. Orchard[1, 3], Zhiyi Xu[1], R. Scott McIvor[3, 4], Adrienne A Brian[5], Bruce R. Blazar[1, 3]

[1] Department of Pediatrics, Division of Pediatric Hematology-Oncology and Bone Marrow Transplantation, University of Minnesota, Minneapolis, Minnesota 55455, USA
[2] Center for Blood Research, Department of Pathology, Harvard Medical School, Boston, MA 02115, USA
[3] Institute of Human Genetics, University of Minnesota, Minneapolis, Minnesota 55455, USA
[4] Department of Laboratory Medicine and Pathology, University Minnesota, Minneapolis, Minnesota 55455, USA
[5] Department of Chemistry and the Cancer Center, University of California, San Diego, La Jolla, CA 92093, USA

Received: 28 March 1993/Accepted: 18 August 1993

Abstract. We determined the expression of intercellular adhesion molecules (ICAM) on neuro-2a cells in order to evaluate whether they were involved in cytolysis of murine neuroblastoma. Fluorescence-activated cell sorting analysis revealed that the control neomycin-resistance-gene-transduced line (neuro-2a/LN) had poor expression of ICAM-1 (mean channel fluorescence, MCF = 3.7). An ICAM-1-positive transfectant of neuro-2a (neuro-2a/ICAM-1+) (MCF = 64.3) was generated to evaluate directly the role of this adhesion molecule in cytolysis. Neuro-2a/ICAM-1+ was more sensitive to LAK killing (69.7% at an effector-to-target ratio of 100:1) compared to neuro-2a/LN (48.6%) (P <0.001). Blocking of neuro-2a/LN and neuro-2a/ICAM-1+ lysis with anti-ICAM-1 monoclonal antibodies (mAbs) did not account for all the LFA-1-dependent killing. These data indicate that even in neuro-2a/ICAM-1+ cells, other LFA-1 ligands participated in the effector-target interaction. Therefore, we examined these cell lines for ICAM-2 expression. Both neuro-2a/LN and neuro-2a/ICAM-1+ lines expressed ICAM-2 (MCF = 16.4 and 16.5). ICAM-2 accounted for the majority of the LFA-1-dependent killing in the ICAM-1-negative target, neuro-2a/LN, while ICAM-1 played a primary role in the cytolysis of the ICAM-1+ transfectant. Inhibition of lysis in the presence of anti-ICAM-1 and ICAM-2 mAbs was comparable to that seen with the addition of anti-LFA-1 mAb, indicating that other LFA-1 ligands were not involved in this system. ICAM-1 expression was associated with decreased in vivo tumorigenicity; mice inoculated with neuro-2a/ICAM-1+ cells had a significantly longer survival compared to those receiving neuro-2a/LN cells (median survival time 35.5 versus 24.5 days) (P <0.001). It is important to note that ICAM-1 transfection of murine neuroblastoma did not alter its metastatic potential. We conclude that transfection of mouse neuroblastoma with ICAM-1 increases its sensitivity to in vitro lysis and reduces its in vivo tumorigenicity. In ICAM-1-negative murine neuroblastoma cells, ICAM-2 plays a primary role in cell-mediated lysis.

Key words: Neuroblastoma – Cytolysis – ICAM-1 – ICAM-2 – Tumorigenicity

Introduction

Neuroblastoma, a tumor of neural crest origin, presents in most patients as a primary paraspinal mass with a widespread pattern of metastases. Conventional multimodality therapy has proven ineffective for the majority of children older than 1 year with disseminated disease. This has resulted in a search for innovative therapeutic approaches. Numerous studies have explored ways to enhance the immune response to this tumor. It is well established that neuroblastoma cells have low expression of major histocompatibility complex (MHC) class I antigens [19, 20] and consequently are resistant to MHC-restricted cytotoxic-T-cell(CTL)-mediated lysis [21]. Interferon γ (IFNγ) induces expression of MHC class I antigens on neuroblastoma lines [19, 22]. This has been shown to increase their sensitivity to CTL-mediated lysis by some investigators but not by others [22, 46].

Cell-adhesion molecules (CAM) are essential in effector-target interactions [23, 26, 30, 36, 43]. Lymphocyte-function-associated antigen (LFA-1), a member of the integrin family, consists of a 180-kDa α subunit (CD11a) and a 95-kDa β subunit (CD18) and plays a major role in leukocyte adhesion [4, 5, 36]. Intercellular adhesion molecule 1 (ICAM-1), the first ligand described for LFA-1, is a member of the immunoglobulin superfamily and has five immunoglobulin-like domains with the binding site for

This work was supported in part by the Children's Cancer Research Fund, the Minnesota Medical Foundation, the Viking Children's Fund and NIH grants PO1-CA-21737, NO1-AI-85002. E. K. is a recipient of the Irvine McQuarrie Research Scholar Award and B. R. B. a recipient of the Edward Mallinkrodt Foundation Scholar Award

Correspondence to: E. Katsanis, University of Minnesota, Box 484 UMHC, 420 Delaware St. S. E., Minneapolis, Minnesota 55455, USA

LFA-1 found on its N-terminal domain [6, 14, 34, 37]. ICAM-1 is expressed by a wide variety of normal cells and heterogeneously by certain tumors. Neuroblastoma cell lines have poor expression of ICAM-1, which may prevent stable conjugate formation with CTL and contribute to their decreased susceptibility to lysis [11, 25]. Cytokines such as IFNγ induce expression ICAM-1 on neuroblastoma lines [11, 12, 25]. Induction of ICAM-1 by IFNγ has been reported by some investigators to enhance sensitivity of neuroblastoma to lysis by MHC-unrestricted lymphokine-activated killer (LAK) cells while others have demonstrated a reduction in killing [12, 13, 25]. More recently, a second ligand for LFA-1 has been described and termed ICAM-2, which has partial homology with ICAM-1 and is made up of two immunoglobulin-like domains [3, 38]. To our knowledge, there are no published studies on whether neuroblastoma cells express ICAM-2 and if this ligand is important in cell-mediated killing of this tumor.

Similar to its human counterpart, murine neuroblastoma has low MHC class I expression inducible by murine IFNγ [8, 33, 42], is resistant to CTL-mediated lysis [2, 29] and sensitive to LAK killing [29, 32, 33]. The importance of CAM, however, in the immune surveillance of murine neuroblastoma has not been previously examined. In this study we transfected the ICAM-1-negative murine neuroblastoma cell line, neuro-2a, with the murine ICAM-1 gene [18, 35] in order to study more directly the role of this adhesion molecule in the interaction between lymphoid effector cells and mouse neuroblastoma targets. Since ICAM-1 expression has been reported to influence tumor progression and dissemination [15, 27], we used a retroperitoneal mouse model [17] to evaluate whether transfection of murine neuroblastoma with the ICAM-1 gene would alter its in vivo tumorigenicity or its metastatic potential. Finally, we used a new anti-(murine ICAM-2) monoclonal antibody (mAb) to examine the relative contribution of this ligand in cytolysis of ICAM-1-positive and -negative neuroblastoma clones. We demonstrate that transfection of murine neuroblastoma with the murine ICAM-1 gene enhances its susceptibility to lysis, reduces its in vivo tumorigenicity and decreases the ICAM-2/LFA-1-dependent killing.

Materials and methods

Murine neuroblastoma transfection and transduction. Neuro-2a (H-2^a), a subclone of C1300 murine neuroblastoma derived in A/J mice (H-2^a) [1, 28] (American Type Culture Collection, Rockville, Md.), was maintained in culture in Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, N.Y.) supplemented with 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 0.5× MEM amino acids (Sigma, St. Louis, Mo.), 1 mM sodium pyruvate (Sigma), 50 μM 2-mercaptoethanol (Sigma) and 10% heat-inactivated fetal bovine serum (Sigma).

A eukaryotic expression vector containing the sequence coding for murine ICAM-1 under transcriptional regulation of the human β-actin promoter [18, 35] and the neomycin phosphotransferase gene was transfected into neuro-2a by using the DNA/calcium phosphate co-precipitation technique [9]. Two days after transfection, the cells were subcultured into medium containing 0.8 mg/ml neomycin analog G418 (Geneticin, Gibco). Resistant colonies were isolated with cloning cylinders (Bellco, Vineland, N.J.), expanded and tested for expression of ICAM-1 by fluorescence-activated cell sorting (FACS) analysis as described below. The expression of ICAM-1 in the neuro-2a clone used in this study (neuro-2a/ICAM-1^+) was tested several times over a period of 6 months and found to be stable.

The retroviral plasmid LN, kindly provided by Dr. A. D. Miller [24] was transfected into the ecotropic packaging line ψ-2. Forty-eight-hour transient supernatants were collected and used to infect PA-317 amphotropic packaging cells (ATCC). Neomycin-resistant colonies were isolated, expanded and viral production assayed by titering on NIH 3T3 tk cells. Viral stocks containing 1.2×10^6 colony-forming units/ml were prepared and used to expose neuro-2a cells at a multiplicity of 5 viral particles/neuro-2a cell in the presence of 8 μg/ml polybrene. After 24 h, exposed cells were subcultured into 0.8 mg/ml G418 and resistant neuro-2a/LN clones isolated.

Evaluation of CAM expression of neuro-2a clones. Neuro-2a clones (neuro-2a/LN and neuro-2a/ICAM-1^+) isolated on the basis of G418 resistance were washed in phosphate-buffered saline (PBS) containing 2% heat-inactivated fetal bovine serum and 0.1% sodium azide (Sigma). A sample containing 1×10^5 cells was placed in each well of 96-well V-bottomed microtiter plates (Nunc, Naperville, Ill.). ICAM-1 and ICAM-2 expression of neuro-2a clones was determined by incubating saturating amounts of mAbs with the cells for 30 min at 4° C. Antibodies used included fluorescein-isothiocyanate(FITC)-labeled anti-(mouse ICAM-1) mAb (clone 3E2; hamster IgG, PharMingen, San Diego, Calif.) and anti-(mouse ICAM-2) (clone 3C4.3; rat IgG2a), which has been found to recognize ICAM-2-transfected COS cells (H. Xu et al. in preparation). The cells were then washed three times and fixed with PBS containing 2% formaldehyde (Polysciences, Warrington, Pa.). A total of 10^4 cells, determined by forward light scatter, were analyzed using a Becton Dickinson FACScan and Consort II software.

ICAM-1 expression of single-cell suspensions from excised tumors was determined as described above with the addition of anti-(mouse Fcγ II receptor) mAb (clone 2.4G2; rat IgG2b) [40] prior to staining with FITC-conjugated anti-ICAM-1. This was done to block Fc receptors of monocytes or natural killer (NK) cells, which may have been present in the tumor suspension.

LAK cell preparation and culture. Spleens from A/J mice were removed under sterile conditions and placed in tissue-culture medium (TCM) consisting of RPMI-1640 medium (Gibco) supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5× MEM amino acids, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol and 10% heat-inactivated fetal bovine serum. The spleens were crushed with the piston of a syringe and spleen fragments gathered in a pipette and filtered through a 100-μm Nytex mesh (Tetko Inc., Elmsford, N.Y.). Splenocytes were washed once in TCM and the red cells removed by hypotonic lysis. Mononuclear cells were washed three times and resuspended in fresh TCM. LAK cultures were generated by incubating 1×10^6 cells/ml in TCM containing 1000 U/ml IL-2 [10]. Cultures were supplemented with IL-2-containing TCM to maintain a cell density of $(0.2-1.0) \times 10^6$ cells/ml.

Cell sorting of LAK cultures. Day-5 LAK cells were washed in PBS containing 2% heat-inactivated fetal bovine serum and 10×10^6 cells/ml were incubated for 3 min with rat anti-(mouse Fcγ II receptor) mAb (clone 2.4G2) [40] to block Fc receptors. The cells were then incubated with saturating amounts of FITC-labeled anti-(Lyt 2) (clone 53-6.7, rat IgG2a, Becton Dickinson, Mountain View, Calif.) for 30 min at 4° C and then washed three times. LAK cells were then positively sorted using a FACS-Star laser flow-cytometry system (Becton Dickinson).

Cytotoxicity assay. ^{51}Cr-release assays were performed as described previously [16]. In brief, neuro-2a target cells [$(1-2) \times 10^6$ in 0.5 ml TCM] were incubated with 500 μCi $Na^{51}CrO_4$ (5 mCi/ml) (Amersham Corporation, Arlington Heights, Ill.) for 1 h at 37° C. Targets were washed three times in TCM and resuspended at a concentration of 2×10^4 cells/ml. Samples of 50 μl (1000 targets/well) were then added to 96-well V-bottomed microtiter plates to which effectors had been previously added in triplicate and serially diluted in TCM to yield effector-to-target ratios from 100:1 to 3.7:1. Spontaneous-release wells contained only

TCM and ^{51}Cr-labeled targets; maximal-release wells contained 2% Triton X-100 (Sigma) and ^{51}Cr-labeled targets. The microtiter plates were centrifuged at 200 *g* for 5 min and incubated at 37° C and 5% CO_2 for 3 h.

The plates were then centrifuged at 200 *g* for 10 min after which 100-µl aliquots of supernatant were harvested into glass scintillation vials (Dynalon, Rochester, N. Y.) and 2.5 ml scintillation fluid added (Cytoscint; ICN Biomedicals, Irvine, Calif.). Radioactivity was counted using a LKB 1216 liquid scintillation counter. Cytotoxicity was determined by the formula:

$$\text{cytotoxicity (\%)} = 100 \times \frac{\text{experimental mean radioactivity (cpm)} - \text{spontaneous-release mean radioactivity (cpm)}}{\text{maximal-release mean radioactivity (cpm)} - \text{spontaneous-release mean radioactivity (cpm)}}$$

Blocking studies. Hybridomas were used to produce monoclonal antibodies for use in blocking experiments. Monoclonal antibodies were purified from ascites by precipitation with 50% ammonium sulfate followed by dialysis against PBS. Blocking studies were performed by blocking Fc receptors on effector cells with 2.4G2 (anti-FcγIIR; rat IgG2b) [40] for 3–5 min followed by incubation of effector cells and/or ^{51}Cr-labeled targets (at an effector-to-target ratio of 100 : 1 or 50 : 1) with anti-(mouse LFA-1 α chain) (clone FD441.8; rat IgG2b) [31], anti-(mouse ICAM-1) (clone BE29G1; rat IgG2a) [18], anti-(mouse ICAM-1) (clone YN1/1; rat IgG2a) [39] and/or anti-(mouse ICAM-2) (clone 3C4.3; rat IgG2a) for 30 min at 4° C and saturating antibody concentrations of 150 µg/ml. The microtiter plates were then centrifuged at 200 *g* for 5 min and incubated at 37° C and 5% CO_2 for 3 h. The plates were then centrifuged at 200 *g* for 10 min after which 100-µl aliquots of supernatant were harvested into glass scintillation vials and 2.5 ml scintillation fluid added. Radioactivity was counted using a LKB 1216 liquid scintillation counter.

Retroperitoneal murine neuroblastoma model. Female A/J (H-2^a) mice, 6–8 weeks old (Jackson Laboratories, Bar Harbor, Me.) were used for the experiments. The animals were housed and fed ad libitum according to University of Minnesota Research Animal Resources guidelines. Neuro-2a/LN and neuro-2a/ICAM-1$^+$ clones maintained in culture in our laboratory were used for tumor induction. 1×10^6 cells in 0.2 ml were inoculated retroperitoneally (r. p.) as described previously [17]. Briefly, prior to r. p. injection, mice were anesthetized i. p. with 1.2–1.4 mg sodium pentobarbital (Nembutal, Abbott, North Chicago, Ill.). The coat over the back and left side was shaved with an Oster small-animal clipper, and the location of the spine, left kidney and spleen was identified under the skin. Tumor cells were carefully inoculated r. p. in the left suprarenal area using a 27-gauge needle. In order to determine whether metastases were present in the livers, spleens and bone marrows of tumor-bearing mice, 5×10^6 cells from these organs (obtained 20 days following r. p. tumor induction) were injected r. p. into healthy A/J mice.

Cytokines. Human recombinant IL-2 with a specific activity of 1.6×10^7 U/mg, was kindly provided by Dr. Maurice Gately from Hoffman LaRoche Inc., Nutley, N. J.

Statistical analysis. Student's *t*-test was used to compare differences in cytotoxicity and mean channel fluorescence of cells. The Kaplan-Meier product-limit method was used to plot the survival of mice inoculated with murine neuroblastoma clones and the log-rank statistic to test differences between groups. Differences in incidence of metastases between groups was assessed with Fisher's exact test.

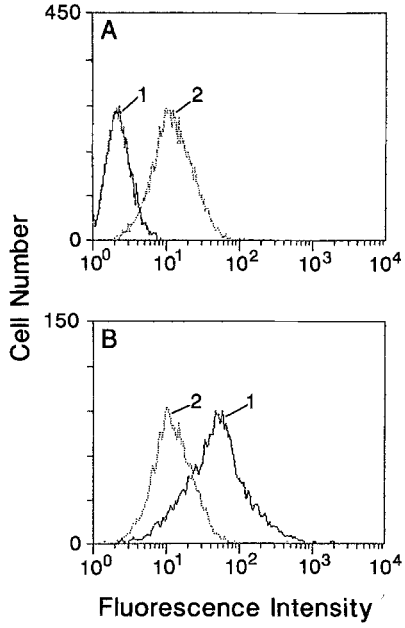

Fig. 1 A, B. Flow-cytometric analysis of (**A**) transduced neuro-2a cells with the neomycin-resistance gene, neuro-2a/LN, and (**B**) transfected neuro-2a with the murine ICAM-1 gene, neuro-2a/ICAM-1$^+$. Cells were analyzed for the expression of ICAM-1 (*1*) and ICAM-2 (*2*)

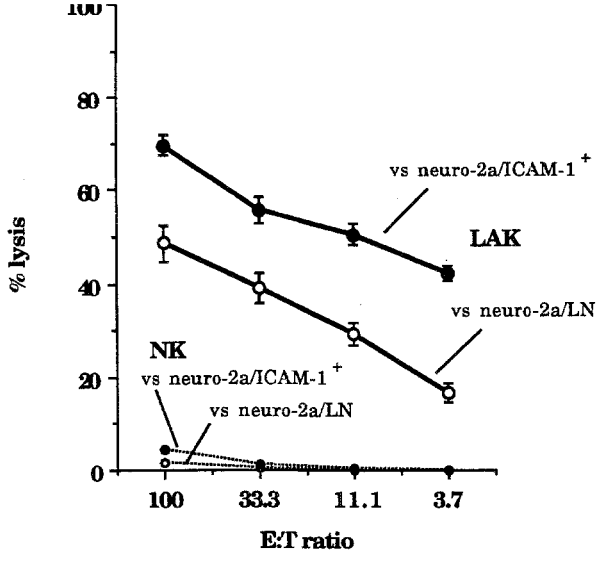

Fig. 2. Lysis of ICAM-1-negative neuro-2a/LN and neuro-2a/ICAM-1$^+$ clones by NK and LAK effectors. Unstimulated fresh A/J splenocytes (*NK*) or A/J splenocytes activated with IL-2 1000 U/ml for 5 days (*LAK*) were used as effectors in 3-h ^{51}Cr release assays. The means and SEM of six experiments are shown. (LAK lysis of neuro-2a/LN versus neuro-2a/ICAM-1$^+$ $P < 0.001$ for each E : T ratio)

Results

Transfection of ICAM-1 into neuro-2a cells increases expression and susceptibility to lysis by LAK cells

We determined the expression of ICAM-1 and ICAM-2 on neuro-2a cells in order to evaluate whether these adhesion molecules were involved in cytolysis of murine neuroblastoma. FACS analysis revealed that the control neuro-2a clone transduced with the neomycin-resistance gene (neuro-2a/LN) poorly expressed ICAM-1 (mean channel fluorescence, MCF = 3.7 versus 1.8 isotype control) and had intermediate levels of ICAM-2 (MCF = 16.4) (Fig. 1). We transfected neuro-2a cells with the murine ICAM-1 gene. The neuro-2a/ICAM-1$^+$ clone had high levels of ICAM-1 (MCF = 64.3) and continued to express intermediate levels of ICAM-2 (MCF = 16.5) (Fig. 1).

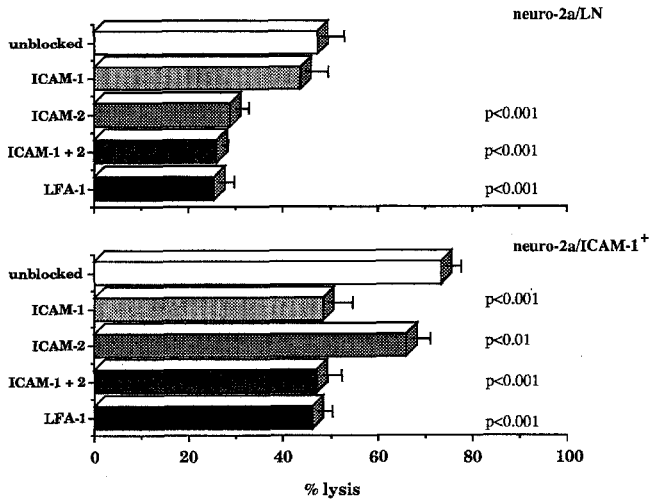

Fig. 3. Inhibition of neuro-2a/LN and neuro-2a/ICAM-1+ lysis by anti-ICAM-1, anti-ICAM-2 and anti-LFA-1 monoclonal antibodies. A/J splenocytes activated with IL-2 1000 U/ml for 5 days (LAK) were used as effectors. Neuro-2a/LN and neuro-2a/ICAM-1+ cells were used as targets. Fc receptors on effector cells were blocked with 2.4G2 mAb for 3–5 min then the effector cells and ^{51}Cr-labeled targets were incubated with 150 μg/ml anti-(mouse ICAM-1) (clone BE29G1 and YN1/1) and/or or anti-(mouse ICAM-2) (clone 3C4.3) or anti-(LFA-1 α chain) (clone FD 441.8) during the 3-h ^{51}Cr-release assay or incubation of effectors with anti-LFA-1 mAb and targets with anti-ICAM mAbs for 30 min prior to the ^{51}Cr-release assay. The means and SD of the percentage lysis at a 100:1 effector-to-target ratio of six replicate wells of a representative experiment are shown

Cytotoxicity assays were then performed to evaluate whether expression of ICAM-1 would alter the sensitivity of neuro-2a to lysis by IL-2-activated and non-activated effector cells. As illustrated in Fig. 2 unstimulated splenocytes were unable to lyse neuro-2a/LN cells. Transfection and expression of ICAM-1 did not enhance susceptibility to NK lysis. However, the neuro-2a/ICAM-1+ clone was significantly (P <0.001) more sensitive than neuro-2a/LN to killing by LAK effectors.

Participation of CAM in cytolysis of ICAM-1-positive and -negative neuro-2a by IL-2-activated cells

Blocking experiments were then performed to evaluate the contribution of LFA-1 and its ligands in the cytolysis of ICAM-1-positive and -negative murine neuroblastoma clones. Fc receptors on effector cells were blocked with 2.4G2 mAb, in order to avoid antibody-dependent cellular cytotoxicity (ADCC) from taking place. We initially evaluated cytolysis by adding the anti-LFA-1 α chain mAb (FD441.8) to effector cells and anti-ICAM-1 (BE29G1 and YN1/1) and/or anti-ICAM-2 mAbs (3C4.3) to the targets. Addition of mAbs (150 μg/ml) in the presence of both effector and target cells yielded similar results.

Anti-LFA-1 mAb significantly (P <0.001) decreased lysis of neuro-2a/LN by LAK cells (Fig. 3). Blocking of ICAM-1 with either BE29G1 or YN1/1 mAb alone or in combination did not significantly reduce killing, suggesting that other LFA-1 ligands participated in LAK-neuro-2a/LN conjugate formation. We therefore examined the role of ICAM-2 in the cytolytic process. Anti-ICAM-2 mAb significantly inhibited killing (P <0.001) indicating that in ICAM-1-negative neuro-2a/LN cells, ICAM-2 is the primary ligand for LFA-1. Inhibition of lysis in the presence of anti-ICAM-1 and anti-ICAM-2 mAbs was comparable to that seen with the addition of anti-ICAM-2 or anti-LFA-1 mAb alone, indicating that ICAM-2 was the only LFA-1 ligand involved in this system. The CD2/LFA-3 interaction did not appear to be important, since saturating concentrations of the anti-CD2 mAb, RM 2.2 [45], did not significantly decrease killing of neuro-2a/LN by LAK cells (data not shown).

Having determined that blocking the ICAM/LFA-1 interaction resulted in decreased lysis of ICAM-1− neuro-2a/LN, we then examined the effect of anti-CAM mAbs on cytolysis of neuro-2a/ICAM-1+ cells (Fig. 3). Lysis of neuro-2a/ICAM-1+ by LAK cells was significantly (P <0.001) inhibited by anti-LFA-1 mAb. Addition of anti-ICAM-1 mAbs (BE29G1 and YN1/1) significantly

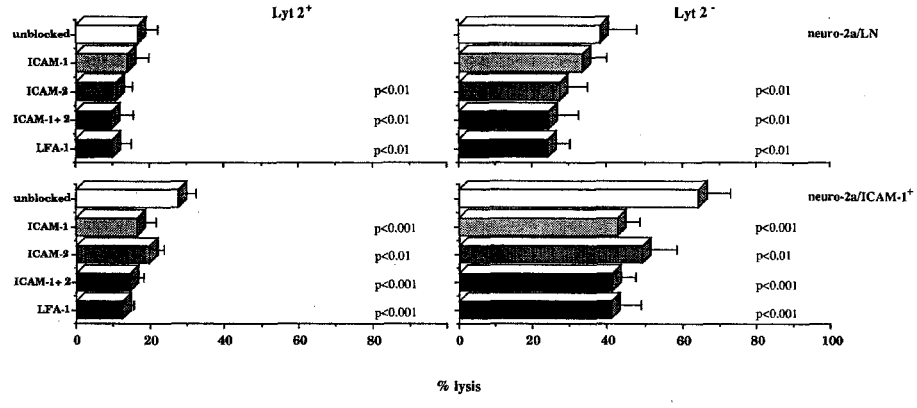

Fig. 4. Inhibition of neuro-2a/LN and neuro-2a/ICAM-1+ by anti-ICAM-1, anti-ICAM-2 and anti-LFA-1 monoclonal antibodies. A/J splenocytes activated with 1000 U/ml IL-2 for 5 days (LAK) were used as effectors. Lyt2+ LAK cells were positively sorted from these cultures just prior to the assay. Neuro-2a/LN and neuro-2a/ICAM-1+ cells were used as targets. Fc receptors on effector cells were blocked with 2.4G2 mAb for 3–5 min then the effector cells and ^{51}Cr-labeled targets were incubated with 150 μg/ml anti-(mouse ICAM-1) (clone BE29G1 and YN1/1) and/or or anti-(mouse ICAM-2) (clone 3C4.3) or anti-(LFA-1 α chain) (clone FD 441.8) during the 3-h ^{51}Cr-release assay. The means and SD of the percentage lysis at a 50:1 effector-to-target ratio of six replicate wells of a representative experiment are shown

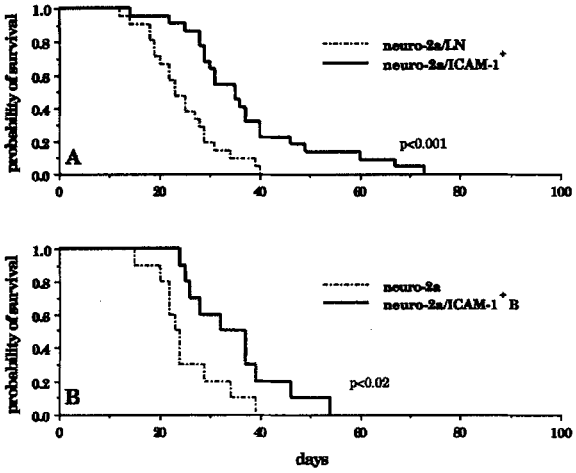

Fig. 5 A, B. Effect of ICAM-1 expression on survival of mice. **A** A/J mice were inoculated r.p. with 10^6 neuro-2a/LN or neuro-2a/ICAM-1+ cells. The combined results of two experiments are shown ($n = 21$ mice/group). Log-rank statistic: $P < 0.001$. **B** A/J mice were inoculated r.p. with 10^6 unmodified neuro-2a or another ICAM-1-expressing clone, neuro-2a/ICAM-1+B, ($n = 10$ mice/group). Log-rank statistic: $P < 0.02$

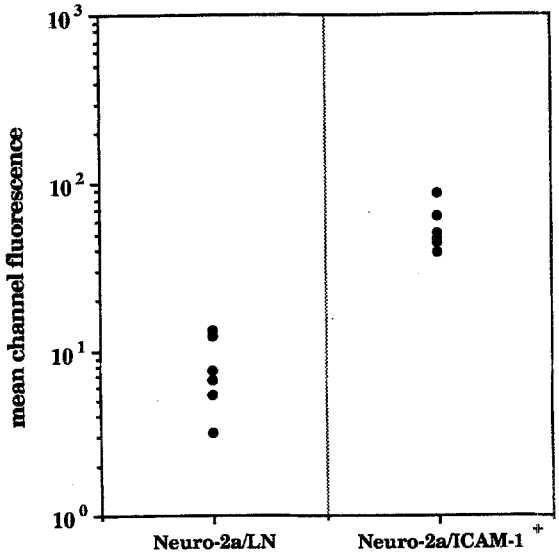

Fig. 6. Flow-cytometric analysis of neuro-2a/LN and neuro-2a/ICAM-1+ tumors resected from mice 20 days following s.c. inoculation. Cells were analyzed for the expression of ICAM-1

($P < 0.001$) decreased killing of neuro-2a/ICAM-1+. Although blocking with anti-ICAM-2 also reduced killing of neuro-2a/ICAM-1+ ($P < 0.01$), the relative contribution of ICAM-2 was not as prominent as in the ICAM-1− neuro-2a/LN cells. There was no difference between blockade with anti-LFA-1 alone and anti-LFA-1 + anti-ICAM-1 + anti-ICAM-2 (data not shown).

We then evaluated the cytolytic function of selected cell subpopulations from within the LAK cultures, to determine if activated T cells or NK cells mediated the majority of the killing (Fig. 4). Positively sorted Lyt2+ cytotoxic T cells were not as potent effectors against neuro-2a targets compared with the population enriched for NK cells (Lyt2− LAK) ($P < 0.001$). Expression of ICAM-1 significantly increased susceptibility to lysis by both Lyt2+ and Lyt2− LAK cells ($P < 0.001$). Blocking studies revealed the same pattern of inhibition as with unsorted LAK, with ICAM-2 accounting for the majority of the LFA-1-dependent killing in the ICAM-1− target and ICAM-1 playing a primary role in the cytolysis of the ICAM-1+ transfectant.

ICAM-1 gene transfer into neuro-2a cells reduces their tumorigenicity

We have recently described a retroperitoneal murine neuroblastoma model which, compared to the subcutaneous model, more closely simulates human disease [17]. Neuroblastoma develops in the majority of children as a retroperitoneal tumor; r.p. administration of neuroblastoma permits the tumor to enlarge locally and metastasize. This site provides an optimal environment for the analysis of the immune surveillance against this tumor. In order to evaluate whether ICAM-1 expression would influence the tumorigenicity of murine neuroblastoma, neuro-2a/LN and neuro-2a/ICAM-1+ cells were injected r.p. into syngeneic A/J mice. As demonstrated in Fig. 5 A, inoculation of 10^6 neuro-2a/LN cells invariably led to death of all mice (median survival time, MST = 24.5 days). ICAM-1 gene transfection and expression decreased the tumorigenicity of neuro-2a with mice receiving 10^6 neuro-2a/ICAM-1+ cells having improved survival (MST = 35.5 days) ($P < 0.001$). Our results were found to be reproducible when another ICAM+ transfectant, neuro-2a/ICAM-1+B was inoculated r.p. and survival compared to mice receiving unmodified neuro-2a (MST = 24 versus 37 days) (Fig. 5 B).

In order to evaluate whether neuro-2a/ICAM-1+ tumors growing in vivo retained their elevated ICAM-1 expression, 20-day-old tumors were excised from mice and surface ICAM-1 expression was compared to neuro-2a/LN tumors. As depicted in Fig. 6, cells from resected neuro-2a/ICAM-1+ tumors expressed higher levels of ICAM-1 (MCF = 55.6) than those from neuro-2a/LN tumors (MCF = 8.1) ($P < 0.001$).

Transfection of neuro-2a with ICAM-1 does not alter its metastatic potential

We have previously shown that adoptive transfer of cells from livers, spleens and bone marrows of neuroblastoma-bearing mice into healthy hosts is more sensitive in detecting micrometastases than microscopic examination of tissue sections [17]. Since ICAM-1 expression has been reported to influence tumor dissemination, we evaluated the propensity of neuro-2a/LN and neuro-2a/ICAM-1+ tumors to metastasize following r.p. inoculation. Adoptive transfer of hepatic single-cell suspensions (5×10^6 cells), obtained from mice with 20-day-old neuro-2a/LN or neuro-2a/ICAM-1+ tumors, into healthy hosts resulted in tumor growth and death of 4/12 and 3/12 of mice respectively (P = NS). Furthermore, r.p. injection of splenic cell suspensions from tumor-bearing mice led to the development of tumors in 2/12 (neuro-2a/LN) and 2/12 (neuro-

2a/ICAM-1+) secondary recipients (P = NS). r.p. inoculation of bone marrow from 12 neuro-2a/LN and 12 neuro-2a/ICAM-1+-bearing mice into healthy hosts failed to result in tumor development after a follow-up period of 100 days. It therefore appears that ICAM-1 expression by murine neuroblastoma cells does not enhance tumor dissemination to distant sites.

Discussion

We have utilized a murine neuroblastoma model to examine the role of CAM in the anti-neuroblastoma immune response. We have shown that, like the human tumor, murine neuroblastoma neuro-2a has poor endogenous expression of ICAM-1. In order to examine directly the involvement of ICAM-1 in cytolysis, we transfected neuro-2a with the murine ICAM-1 gene and generated a high-ICAM-1-expressing clone. We have clearly demonstrated that ICAM-1 transfection results in enhanced susceptibility to lysis by IL-2-activated effectors including CD8+ T cells and NK cells. Our results are consistent with observations by Naganuma et al., who reported that incubation of neuroblastoma lines with IFNγ increased ICAM-1 expression and was associated with increased killing by LAK effectors [25], but are in contrast to those of Gross et al., who demonstrated decreased susceptibility to LAK lysis of various human neuroblastoma lines following treatment with IFNγ [12]. Since IFNγ has complex effects on modulation of different accessory molecules on tumor cells, transfection of neuro-2a with the ICAM-1 gene allowed us to analyze the role of ICAM-1 separately.

Based on ICAM-1 blockade studies in a number of tumor cell lines, it would appear that LFA-1-dependent killing involves other ligands such as ICAM-2. The murine ICAM-2 gene has recently been cloned and found to be highly homologous to the human molecule [44]. With the use of a new anti-(murine ICAM-2) mAb (Xu et al. in preparation), we examined the involvement of ICAM-2 in the cytolysis of ICAM-1-positive and -negative murine neuroblastoma clones. We found that ICAM-2 plays an important role in the LFA-1-dependent killing of ICAM-1− neuro-2a/LN cells, while ICAM-1 assumed the primary role in cytolysis of the neuro-2a/ICAM-1+ line. Although several LFA-1-dependent, ICAM-1-independent cell interactions have been accounted for by ICAM-2, a third ligand for LFA-1 has recently been described [41]. However, other LFA-1 ligands did not appear to be critical in our system since blocking with anti-ICAM-1 and anti-ICAM-2 mAbs accounted for all the LFA-1-dependent killing of both ICAM-1-positive and -negative neuro-2a clones. Of note in our studies is that LFA-1 or ICAM-1 and ICAM-2 blockade of the neuro-2a/ICAM-1+ line was associated with higher killing when compared to the neuro-2a/LN clone. The reason for this is unclear to us at this time but it was found to be reproducible in six experiments performed using saturating mAb concentrations of 150 μg/ml.

There have been conflicting reports on the association between ICAM-1 and tumor progression and dissemination. Recent studies have indicated that ICAM-1 expression of melanoma correlates with lesion thickness and metastases [15, 27]. The disease-free survival of patients with ICAM-1− melanomas was significantly better than those with ICAM-1+ primary lesions [27]. Metastatic deposits were found to have higher levels of ICAM-1 than the primary tumors [15, 27]. It has been suggested that ICAM-1 expression by melanoma cells results in their adhesion to LFA-1-bearing leukocytes, which may facilitate tumor dissemination [15]. Both neuroblastoma and melanoma are tumors of neuroectodermal origin. In contrast to melanoma cells, ICAM-1 has been found to be preferentially expressed on low-stage well-differentiated neuroblastoma specimens [7, 12]. Furthermore, in one study of 51 clinical neuroblastoma specimens, ICAM-1 expression was restricted to N-*myc*-negative tumors [7]. To assess further the influence of ICAM-1 on tumorigenicity, we compared the survival of mice inoculated with ICAM-1-positive and negative neuro-2a cells. ICAM-1 expression was associated with improved outcome. This may in part be due to the enhanced susceptibility to lysis of ICAM-1+ neuro-2a cells. Moreover, we demonstrated that mice inoculated with ICAM-1-transfected neuroblastoma cells did not have an increased incidence of metastases. We have also examined the expression of ICAM-1 in metastatic neuro-2a lesions. Tumor cells obtained from metastases did not have increased expression of ICAM-1 when compared to cells from primary neuro-2a tumors (unpublished data).

In summary, we have demonstrated that transfection of mouse neuroblastoma with ICAM-1 increases its sensitivity to in vitro lysis and reduces its in vivo tumorigenicity without enhancing metastatic potential. Moreover, the importance of ICAM-2 in the cytolytic process of ICAM-1-negative murine neuroblastoma cells has been documented.

Acknowledgements. The authors wish to thank Betsy Barrett and Keith Gorden for their invaluable technical assistance.

References

1. Augusti-Tocco G, Sato G (1969) Establishment of functional clonal lines of neurons from mouse neuroblastoma. Proc Natl Acad Sci USA 64: 311–315
2. Choi SH, Reynolds JV, Ziegler MM (1989) Systematic analysis of the immunoregulation of murine neuroblastoma. J Pediatr Surg 24: 15–20
3. de Fougerolles AR, Stacker SA, Schwarting R, Springer TA (1991) Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J Exp Med 174: 253–267
4. Dustin ML, Springer TA (1988) Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J Cell Biol 107: 321–331
5. Dustin ML, Springer TA (1989) T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature 341: 619–624
6. Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA (1986) Induction by IL-1 and interferon-γ: tissue distribution, biochemistry and function of a natural adherence molecule (ICAM-1). J Immunol 137: 245–254
7. Favrot MC, Combaret V, Goillot E, Tabone E, Bouffet E, Dolbeau D, Bouvier R, Coze C, Michon J, Phillip T (1991) Expression of leukocyte adhesion molecules on 66 clinical neuroblastoma specimens. Int J Cancer 48: 502–510

8. Gopas J, Itzhaky D, Segev Y, Salberg S, Trink B, Isakov N, Zisman-Rager B (1992) Persistent measles virus infection enhances major histocompatibility complex class I expression and immunogenicity of murine neuroblastoma cells. Cancer Immunol Immunother 34: 313–320
9. Graham FL, der Eb AJV (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52: 456–467
10. Grimm EA, Mazumder A, Zhang H, Rosenberg SA (1982) Lymphokine activated killer phenomenon: lysis of natural killer-resistant fresh solid tumor cells by interleukin-2 activated autologous human peripheral blood lymphocytes. J Exp Med 155: 1823–1830
11. Gross N, Carrel S, Beck D, Favre S (1991) Cell adhesion molecules expression and modulation on human neuroblastoma cells. Adv Neuroblastoma Res 3: 293–299
12. Gross N, Favre S, Beck D, Meyer M (1992) Differentiation-related expression of adhesion molecules and receptors on human neuroblastoma tissues, cell lines and variants. Int J Cancer 52: 85–91
13. Handgretinger R, Kimmig A, Lang P, Daurer B, Kuci S, Bruchelt G, Treuner J, Niethammer D (1989) Interferon-gamma upregulates the susceptibility of human neuroblastoma cells to interleukin-2-activated natural killer cells. Nat Immun Cell Growth Regul 8: 189–196
14. Horley KJ Carpenito C, Baker B, Takei F (1989) Molecular cloning of murine intercellular molecule (ICAM-1). EMBO J 8: 1889–1896
15. Johnson JP, Stade BG, Holzmann B, Schwable W, Riethmuller G (1989) De novo expression of intercellular-adhesion molecule 1 in melanoma correlates with increase risk of metastases. Proc Natl Acad Sci USA 86: 641–644
16. Katsanis E, Bausero MA, Ochoa AC, Loeffler CM, Blazar BR, Leonard AS, Anderson PM (1991) Importance in timing of cyclophosphamide on the enhancement of interleukin-2 induced cytolysis. Cancer Immunol Immunother 34: 74–78
17. Katsanis E, Blazar BR, Bausero MA, Gunther R, Anderson PM (1994) Retroperitoneal inoculation of murine neuroblastoma cells results in a reliable model for evaluation of anti-tumor immune response. J Pediatr Surg (in press)
18. Kuhlman P, Moy VT, Lollo BA, Brian AA (1991) The accessory function of murine intercellular adhesion molecule-1 in T lymphocyte activation. Contributions of adhesion and co-activation. J Immunol 146: 1773–1782
19. Lampson LA, Fisher CA (1984) Weak HLA and β_2-microglobulin expression of neuronal cell lines can be modulated by interferon. Proc Natl Acad Sci USA 81: 6476–6480
20. Lampson LA, Fisher CA, Whelan JP (1983) Striking paucity of HLA-A, B, C and β_2-microglobulin on human neuroblastoma cell lines. J Immunol 130: 2471–2478
21. Main EK, Lampson LA, Hart MK, Kornbluth JK, Wilson DB (1985) Human neuroblastoma cell lines are susceptible to lysis by natural killer cells but not cytotoxic T lymphocytes. J Immunol 135: 242–246
22. Main EK, Monos DS, Lampson LA (1988) IFN-treated neuroblastoma cell lines remain resistant to T cell-mediated allo-killing, and susceptible to non-MHC-restricted cytotoxicity. J Immunol 141: 2943–2950
23. Makgoba MW, Sanders ME, Luce GEG, Gugel EA, Dustin ML, Springer TA, Shaw S (1988) Functional evidence that intercellular adhesion molecule-1 (ICAM-1) is a ligand for LFA-1 dependent adhesion in T cell-mediated cytotoxicity. Eur J Immunol 18: 637–640
24. Miller AD, Rosman GJ (1989) Improved retroviral vectors for gene transfer and expression. Biotechniques 7: 980–990
25. Naganuma H, Kiessling R, Patarroyo M, Hansson M, Handgretinger R, Gronberg A (1991) Increased susceptibility of IFN-γ treated neuroblastoma cells to lysis by lymphokine-activated killer cells: participation of ICAM-1 induction on target cells. Int J Cancer 47: 527–532
26. Nakamura T, Takahashi K, Fukazawa T, Koyanagi M, Yokoyama A, Kato H, Yagita H, and Okumura K (1990) Relative contribution of CD2 and LFA-1 to murine T and natural killer cell functions. J Immunol 145: 3628–3634
27. Natali P, Nicotra MR, Cavaliere R, Bigotti A, Romano G, Temponi M, Ferrone S (1990) Differential expression of intercellular adhesion molecule 1 in primary and metastatic melanoma lesions. Cancer Res 50: 1271–1278
28. Olmsted B, Carlson K, Klebe R, Ruddle F, Rosenbaum J (1970) Isolation of microtubule protein from cultured mouse neuroblastoma cells. Proc Natl Acad Sci USA 65: 129–136
29. Reynolds JV, Shou JS, Choi H, Sigal R, Ziegler MM, Daly JM (1989) The influence of natural killer cells in neuroblastoma. Arch Surg 124: 235–239
30. Robertson MJ, Caligiuri MA, Manley TJ, Levine H, Ritz J (1990) Human natural killer cell adhesion molecules. Differential expression after activation and participation in cytolysis. J Immunol 145: 3194–3201
31. Sarmiento M, Dialynas DP, Lancki DW, Wall KA, Lorber MI, Loken MR, Fitch FW (1982) Cloned T lymphocytes and monoclonal antibodies as probes for cell surface molecules active in T-cell mediated lysis. Immunol Rev 68: 135–169
32. Sigal RK, Reynolds JV, Markmann JF, Shou J, Evantash E, Ziegler M, Naji A, Daly J (1988) Upregulation of MHC class I: effect on growth and LAK sensitivity of neuroblastoma. Surg Forum 39: 572–575
33. Sigal RK, Lieberman MD, Reynolds JV, Shou J, Ziegler MM, Daly JM (1991) Low dose interferon gamma renders neuroblastoma more susceptible to interleukin-2 immunotherapy. J Pediatr Surg 26: 389–396
34. Simmons D, Makgoba MW, Seed B (1988) ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Nature 331: 624–627
35. Siu G, Hedrick SM, Brian AA (1989) Isolation of the murine intercellular adhesion molecule 1 (ICAM-1) gene. ICAM-1 enhances antigen-specific T cell activation. J Immunol 143: 3813–3820
36. Springer TA (1990) Adhesion receptors of the immune system. Nature 346: 425–434
37. Staunton DE, Marlin SD, Stratowa C, Dustin ML, Springer TA (1988) Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell 52: 925–933
38. Staunton DE, Dustin ML, Springer TA (1989) Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature 339: 61–64
39. Takei F (1985) Inhibition of mixed lymphocyte response by rat monoclonal antibody to a novel murine lymphocyte activation antigen (MALA-2). J Immunol 134: 1403–1407
40. Unkeless J (1979) Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J Exp Med 152: 1048–1069
41. Vazeux R, Hoffman PA, Tomita JK, Dickinson ES, Jasman RL, St John T, Gallatin WM (1992) Cloning and characterization of a new intercellular adhesion molecule ICAM-R. Nature 360: 485–488
42. Watanabe Y, Kuribayashi K, Miyatake S, Nishihara K, Nakayama E, Taniyama T, Sakata T (1989) Exogenous expression of mouse interferon γ cDNA in mouse neuroblastoma C1300 cells results in reduced tumorigenicity by augmented anti-tumor immunity. Proc Natl Acad Sci USA 86: 9456–9460
43. Webb DSA, Mostowski HS, Gerrard TL (1991) Cytokine-induced enhancement of ICAM-1 expression results in increased vulnerability of tumor cells to monocyte-mediated lysis. J Immunol 146: 3682–3686
44. Xu H, Tong IL, Fougerolles AR de, Springer TA (1992) Isolation, characterization and expression of mouse ICAM-2 complementary and genomic DNA. J Immunol 149: 2650–2655
45. Yagita H, Nakamura T, Karasuyama H, Okumura K (1989) Monoclonal antibodies specific for murine CD2 reveal its presence on B as well as T cells. Proc Natl Acad Sci USA 86: 645–649
46. Zier KS, Pierson GR, Brown V (1990) Susceptibility of human neuroblastoma cell lines to cytotoxic T lymphocyte-mediated lysis. J Neuroimmunol 28: 153–160