Influence of Primary Tumor Site on Host Anti-Tumor Immunity

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Stage IV-S neuroblastoma, characterized by a primary tumor plus disseminated tumors in liver, skin and bone marrow, has a favorable clinical prognosis when compared to metastatic Stage IV neuroblastoma. This favorable outcome also characterized mice receiving tumor transplants to these "IV-S" sites. We report the testing of the hypothesis that enhanced anti-tumor immunity in "IV-S" site neuroblastoma recipients explains this improved survival. A million murine C1300 neuroblastoma cells were inoculated into 256 A/J mice to either "IV-S" sites of skin, liver, peritoneal cavity, or to the disseminated stage "IV" sites of subcutaneous tissue, muscle, kidney and lung. After 21 and 28 days of tumor growth, spleen cells from tumor bearing mice were harvested and analyzed by a 51 Cr release lymphocytotoxicity assay. Cytotoxic T cell activity was consisitently higher at day 28 than day 21. In the liver and in the peritoneal cavity, cytotoxic T cell activity was higher than in other organs, and at day 28 these values were significantly higher than Stage "IV" sites. On the other hand, skin is not a immunologically privileged site in vivo study.

Key Words: Neuroblastoma, anti-tumor immunity

In 1971, Evans and associates proposed a new staging classification for neuroblastoma and introduced the term, stage "IV-S". Stage "IV-S" sites are frequent metastatic foci of neuroblastoma, but these metastatic involvements are often reversible and such patients have a favorable long term survival (Evans et al. 1971). No other tumor has such a high rate of spontaneous regression. The stage "IV-S" category includes the group of patients with disseminated disease who have small primary tumors and distant foci in the liver, skin and bone marrow. Reports by Evans and associated have recounted tumor deposits in the pleura, pancreas, and bowel serosa as well (Evans et al. 1980). It is felt by many that the cure of neuroblastoma is the result of body's natural immune response.

Indeed, in stage "IV-S" neuroblastoma no chemotherapy is used because it is felt that it would interfere with the patient's own ability to produce antibodies against the tumor, and chemotherapy causes a high mortality rate because of complication. With use of this murine neuroblastoma model and normal liver cell, it was previously reported that whole liver cell and subcellular soluble factor immunoregulatory interactions are operative in determining the immunogenecity of tumor inoculation.

We now report the difference of tumor specific immune response between stage "IV-S" sites and stage "IV" sites, and how this immune response changes with time.

MATERIALS AND METHODS

Animals

A/J mice were purchased from Wonjaryuk Hospital, Seoul, Korea. Male animals, 6-8 weeks of age, weighing 20-25 grams were used and a total of 256 animals were studies. They were maintained on

commercial food pellets and tap water.

Tumors

C1300 neuroblastoma arose spontaneously in the A/J mice and had behavior characteristics not unlike the human tumor. It was maintained by serial transplantation in A/J mice and by serial passage in tissue culture in the RPMI 1640 medium (Mediatech, Washington, DC) containing 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY) and 0.03% fresh glutamine (Mediatech).

Tumor induction

C1300 neuroblastoma cells were harvested from a monolayer culture flask and cell viability was determined by dye exclusion using 0.16% trypan blue solution (Eastman Kodak Co. Rochester, NY). Tumors were able to be induced 100 percent of time using a dose 1×10^6 tumor cells and this was chosen as standard for experiments. 256 6-8 weeks old A/J mice were inoculated in our laboratory with 1×10^6 neuroblastoma cells. The 256 mice were divided into fourteen subgroups. Each experimental subgroup consisted of sixteen mice, and there were two control goups containing thirty-two mice. Tumor cells were injected intradermally, intraperitoneally, subcutaneously, intramuscularly, and intrarenally. Liver metastasis was made by subcapsular inoculation and lung metastases was induced by tail vein injection.

Splenocyte suspensions

Spleens were removed aseptically, placed in complete medium(CM), and gently crushed with the rough surface of micro slide. Then, the aspirated cell suspension was placed into a 10 ml syringe, and allowed to sediment in an upright position for 10 minutes. Cells were centrifuged for 10 minutes at 500 G, washed once with CM, and then resuspended to a desired concentration of 2.5×10^7 cells/mL.

The complete medium consisted of RPMI 1640 with 10% heat-inactivated fetal calf serum, 5×10^{-5} M 2-Mercaptoethanol (Sigma, St. Louis, MD), 2 mM Glutamine, 1 mM Sodium pyruvate (Sigma), 0.1 mM Nonessential Amino Acid (Gibco), 100 μg/mL Penicillin (sigma), and 100 μg/mL Streptomycin (Sigma).

In Vitro generation of cytotoxic T lymphocytes

C1300 neuroblastoma cells were harvested from a monolayer culture flask as a single cell suspension. 25 μg of mitomycin C was added per mL of cell suspension. After the living cells were counted with a 0.16% trypan blue dye exclusion, they were resuspended at a concentration of 1×10^6 cells/mL. One mililiter of CM containing 2.5×10^7 viable splenocytes was added to each 25 cm² Corning Tissue culture flask (No. 2500). One mililiter of CM containing 1×10^6 viable irradiated C1300 neuroblastoma cells and 10 mL of CM were also added to each flask. After six days of incubation 37°C with 5% CO_2, cells were harvested and counted by using 0.16% trypan blue, then resuspended in CM.

Chromium release assay of cytotoxicity
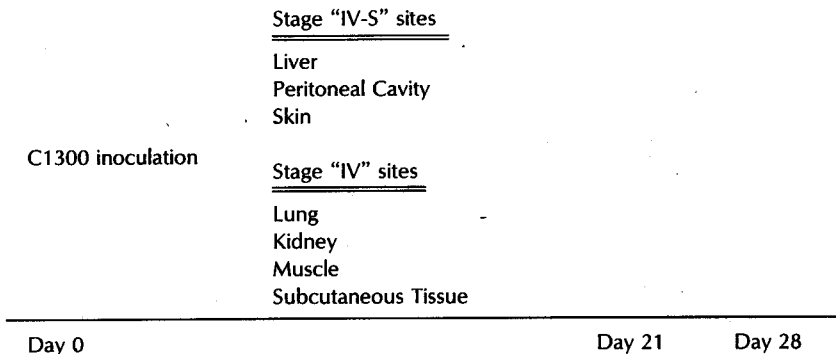
A 4-hour chromium release assay was performed.

Stage "IV-S" sites
Liver
Peritoneal Cavity
Skin

C1300 inoculation

Stage "IV" sites
Lung
Kidney
Muscle
Subcutaneous Tissue

Day 0   Day 21   Day 28

Fig. 1. Flow diagram of experimental protocol. At day 0' all the mice were inoculated with 10⁶ C1300 neuroblastoma either at the "IV-S" sites or at the disseminated "IV" sites. At day 21 and 28, animals were sacrificed and aslyzed lymphocytotoxicity by ⁵¹Cr-release assay.

Various ratios of effector cells were plated in 96 well round-bottomed plates (Linbrochemical Co, Hamden, CT) containing 10⁴ viable C1300 neuroblastoma tumor target cells per well. Plates were then centrifuged at 80 G for 5 minutes, incubated at 37°C for 4hr and then recentrifuged at 500 G for 10 minutes. The supernatants were harvested by using SCS harvesting frames and SCS transfer tube strips (Skatron, Lier, Norway). The percentage of specific lysis was calculated by

$$\frac{\text{Sample counts-Background counts}}{\text{Total counts-Background counts}} \times 100$$

Total counts were released by 0.1 N Triton X, and background counts by CM. In all experiments, the background counts were less than 30% of total counts. With use of the definition of the lytic unit-30 (L.U-30), and arbitrarily defined unit: the number of lymphocytes required to achieve 30% lysis of 10 ×10³ 51 Cr labeled target cell within 4hr, we calculated cytotoxitity again and compare the various groups.

Experimental protocol

Fig. 1 summarized the flow diagram of these ex-periments. At day 0, murine C1300 neuroblastoma was inoculated in mice to either the "IV-S" sites of skin, liver, peritoneal cavity, or to the disseminated stage "IV-S" sites of subcutaneous tissue, muscle, lung and kidney. After 21 and 28 days of tumor growth, mice were harvested and analyzed by 51 Cr-release assay. At the same time, the gross metastasis pattern was reviewed by the India Ink injection technique. Tissue taken at autopsy was fixed in 10% formalin for 24 hours. Soft tissue were sectioned and stained with Hematoxylin and Eosin.

Statistic analysis

The Wilcoxon rank-sum test was done to assess differences of cytotoxicity of sensitized cells between day 21 and day 28. The paired t test was used to compare the differences of cytotoxicity between the subgroups.

RESULTS

Cytotoxicity of sensitized T cells

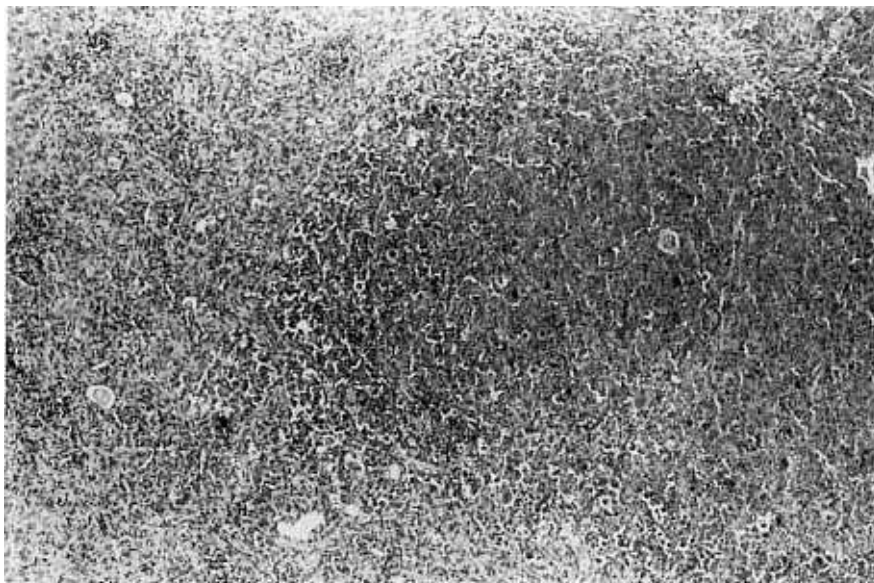At day 21, there were no significant differences in

Fig. 2. *Murine neuroblastoma in the liver parenchyme: The tumor consists of round cells with numerous mitotic figures. There are no dendritic processes which suggests highly the tumor is malignant. Nuclei are hyperchromatic. Dense infiltration of lymphocytes and macrophages are also seen. Pressure deformation and tumor growth growth within the sinusoid can be observed.*

cytotoxicity between Stage "IV-S" sites and Stage "IV-S" sites but even at this stage, the liver and peritoneal cavity showed higher cytotoxcicity than the other organ sites. Cytotoxicity of day 28 was significantly higher than that of day 21 ($p < 0.05$) in liver, pertoneal cavity, skin and lung. In other words, cytotoxicity increased significantly in all

Stage "IV-S" sites but was unchanged in all Stage "IV" sites except the lung. At day 28, the cytotoxicity of liver and peritoneal cavity were higher than those other organs. Those were statistically significant (Table 1).

Macroscopic and microscopic findings

The India ink injection technique was used for lung and liver metastasis. In liver metastasis, India ink was injected via the tail vein, and the liver was removed and examined for gross metastasis. In the lung metastasis, India ink was injected through the trachea, and the lung was harvested for gross counting. Both in the lung and the liver, metastatic patterns were not multiple. There were several metastatic foci which were huge in size. Other than in the liver and lung metastasis models, there were no spontaneous metastases. Significant enlargement of the spleen was found in all tumor bearing animals. Microscopically, the tumor consisted of round cells with numerous mitotic figures, and nuclei of round cells were hyperchromatic. These round cells lacked a dendritic process. Also, the tumor demonstrated many necrotic areas with mixed granular, acellular debris. There were dense infiltrations of lymphocytes and macrophages. In no occaison

Table 1. Cytotoxicity of tumor bearing mice at day 21 and day 28

	Day 21	Day 28
Stage "IV-S" sites		
Liver	2.36*	3.24**
Peritoneal Cavity	2.30	3.23**
Skin	1.75	2.73
Stage "IV" sites		
Lung	1.51	2.68
Kidney	2.02	2.10
Muscle	2.16	2.45
Subcutaneous Tissue	2.00	2.33

*Lytic unit-30
**$p < 0.05$

Fig. 3. Renal tumor in renal cortex: Central necrosis is seen in the middle of tumor. The tumor demonstrates necrotic area with mixed granular, acellular debris. There are dense infiltrations of lymphocytes and macrophages in the tumor mass. Pressure deformation in the normal renal parenchyme can be ovserved.

were tumor emboli found even though the tumor frequently invaded surrounding tissues. The liver tumor grew within the sinusoid, where it caused pressure deformation. There were many metastatic foci in pulmoary vessels; this was natural because the tumor cells were given via the tail vein. However, tumor growth in the alveolar septa was also found which meant a lymphatic spread of this tumor. The kidney tumors were made by cortical tumor cell injection, so these caused pressure deformation of the calyciopelvic system.

DISCUSSION

Neuroblastoma is the most common solid tumor in childhood and represents 7-10% of all cases of childhood cancer. But it results in 8.4-15% of cancer deaths in children (Stokes et al. 1984; Lopez-Ibor et al. 1985; Keogh et al. 1976). Even though three-fourths of patients have disseminated disease at the time of presentation, they still have a good prognosis. Small neuroblastoma foci are found in approximately one out of every fifty to one hundred births, but the actual incidence of neuroblastoma is about 1 in 10,000 (Bill 1971). These observations strongly suggest that immune system of the host strongly affects the host-neuroblastoma relationship.

The cells from the neural crest, called sympathogonia, may develop into ganglion cells, pheochromocytes, or neurofibrous tissue. Tumors from these tissues are neuroblastoma, ganglioneuroblastoma, ganglioneuroma, pheochromocytoma, and neurofibroma (LopezIbor et al. 1985). Kudson suggested that Stage IV-S neuroblastoma is not in facta malignant timor, but rather a hyperplastic nodule of mutant cells lacking a second event present in typical neuroblastoma cells. A further event (second hit) can transform Stage IV-S neuroblastoma lesions into malignant neuroblastoma (Knudson et al. 1980).

It has been suggested by other authors that patients with bone marrow metastasis. be excluded from Stage IV-S classification because bone marrow involvement with tumor reduces the otherwise favorable outlook for patients with stage IV-S disease (Grosfeld et al. 1978). Recently, Stage IV-S neuroblastoma was further divided into two risk categories based on age and the combination of metastatic site present at diagnosis (Stephenson et al. 1986). Four prognostic groups were suggested in Stage IV neuroblastoma using histology, Children's Cancer Study Group staging, and the presence of positive lymph nodes (O'Neill et al. 1985; Shimada et al. 1984). Based on these classification, we decided to make Stage IV-S model by injecting tumor cells in the liver, peritoneal cavity, and skin. Stage IV models were produced by injecting tumor cells into kidney, lung, and subcutaneous tissue.

Anti-tumor responses in vivo have several cytolytic phenomena, including compliment-dependent antibody-mediatied cytotoxicity, T-cell cytotoxicity, cell killing by macropgage, and antibody-dependent cellular cytotoxicity. Among these, cytotoxic immune activity of lymphocytes against the patient's own tumor is most important. This is mediated by small lymphocytes (Bill 1971; Byfield et al. 1976). Cytotoxicity mediated by small lymphocytes can be measured by the Chromium-release assay described above. At day 28, cytotoxicities of liver and peritoneal tumor bearing animals were significantly higher than that of Stage "IV" sites. So it was speculated that improved survival in Stage "IV-S" is closely related to this increased cytotoxicity. Although host immunity may be intact initially, as the tumor progressively grows and disseminates, general impairment of the immune responsible for neutralizing the immune response of the host. Such factors includes suppressor cells and their products, acute phase reactants, and suppressor substances produced by tumors (Roth et al. 1983). By this in vitro cytotoxicity test, it was demonstrated that lymphocytotoxicity is not influenced by tumor growth; it is augumented in liver, peritoneal cavity, skin and lung. So impairment of the immune system is not because of impairment of lymphocytotoxicity but because of increased acitvity of suppressor cells and humoral factors.

Though the growth characteristics, histology and metastases in this tumor-host system are similar to the human pattern, a shortcoming of this tumor system is the lack of either spontaneous maturational change or spontaneous cure. C1300 murine neuroblastoma proved to be highly immunogenic by immnization-excision-challenge assay, mixed lymphocyte culture and Chromium release assay (Karpehet al. 1987; Ziegler et al. 1986; Choi et al. 1988). On the other hand, most human tumors are regarded as minimally or non-immunogenic. Human neuroblastoma is susceptible to lysis by natural killer cells not by cytotoxic T lymphocytes (Main et al. 1985). It is also demonstrated that C1300 murine neuroblastoma is sensitive to natural killer cells (Choi et al. 1988). Stage IV-S neuroblastoma can be inherited (Knudson et al. 1980) and lymphocytes of

68% individuals among fathers, mothers, and siblings showed positive activity in colony inhibition test (Bill 1971; Hellstrom et al. 1970). Electro-coagulation potentiates the immunogenicity of the neuroblastoma, potentiates the host antitumor immune response to residual primary tumor, and potentiates the host antitumor immune response to distant autochthonous neuroblastoma (Ziegler et al. 1980).

It is postulated that the liver may contain an inhibitor which suppresses the lymphocyte and can be demonstrated when the histocompatibility is weak (Pfeffermann et al. 1976). Former experiments using liver cells and C1300 neuroblastoma demonstrated that tumor cells admixed with liver in vitro are rendered less immunogenic (Ziegler et al. 1979; Ziegler et al. 1981). This findings are also reported for alloantigen. The liver may posses the potential to alter the antigenecity of allografts (Mandel et al. 1965). The liver can induced a from of afferent immunologic blockade to a tumor residing within it, and such immunologic privilege may account for the high frequency of neuroblastoma liver metastases (Ruben et al. 1983).

Histologic prognostic factors include amount of stroma, degree of maturation and nuclear morphology (mitosis and Karyorrhexis) (Simada et al. 1984). Together with this histologic evaluation, other factors are important in humans: patient's age at diagnosis, stage at diagnosis and primary tumor location, NSE (neuron specific enolase), ferritin, VMA/HVA ratio (Evans et al. 1987). Ratio of cells in the S and G2/M phases of the cell cycle determined by flow cytometric DNA analysis is highly correlated with clinical outcome. This is a better indicator of prognosis than nuclear morphology (Gansler et al. 1986). Tumor emboli could not be found in histologic slides, but it was reported in C1300 neuroblastoma that tumor emboli in the circulating blood were demonstrateed by Ficoll-Hypaque gradient. Tumor growth in the alveolar septa in intravenously injected mice supported former findings that C1300 neuroblastoma metastasize by way of the blood vessels and the lymphatic channel (McAlack et al. 1977). Spontaneous metastases were rarley found in C1300 neuroblastoma (Arima et al. 1973). Mouse death is typically caused by local tumor growth and is not the result of metastases. As seen in the liver and kidney, the tumor caused pressure deformation in its later stages.

All patients with Stage IV-S neuroblastoma and skin involvement showed most favorable prognosis (Grosfeld et al. 1978). Skin is not a immunologically previleged site, so this occurrence implicates an unusual tumor-host relationship.

These data showed increased lymphocytotoxiciity in Stage IV-S, especially in the liver and the peritoneal cavity. This increased cytotoxicity reflects a high host-antitumor immune response in Stage IV-S. There were no spontaneous metastases in the C1300 tumor model.

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