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NATURAL KILLER CELLS IN INFLAMMATORY LESIONS AND TRANSPLANTED TUMORS IN MOUSE SKIN

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ABSTRACT

The mode of natural killer (NK) cell migration to the sites of inflammation and transplanted tumors was investigated by using dry ice for physical irritation and 1-fluoro-2,4 dinitrobenzene (DNFB) for chemical irritation in mouse ear. In experiments with transplanted tumors, NK cell sensitive tumor cells (RL♂1) and insensitive tumor cells (p815) were transplanted into the ears of C3H and BALB/c mice, respectively. Employing a polyclonal rabbit antiserum against asialoGM1 (GA1), and a monoclonal rat antiserum against Thy-1 in an immunohistochemical double-staining technique, we enumerated the number of Thy-1-positive and asialoGM1-positive (Thy-1$^+$GA1$^+$) cells and Thy-1-negative and asialoGM1-positive (Thy-1$^-$GA1$^+$) cells at various times of irritation.

Following physical irritation, Thy-1$^-$GA1$^+$ cells (108.8 ± 4.5/mm^2 at 24 h and 71.2 ± 3.8/mm^2 at 48 h) were found in the epidermis, whereas Thy-1$^+$GA1$^+$ cells were not found. In delayed-type skin reaciton by DNFB, Thy-1$^-$GA1$^+$ cells (87.1 ± 5.8/mm^2 at 24 h and 60.7 ± 2.9/mm^2 at 48 h) and Thy-1$^+$GA1$^+$ cells (26.4 ± 3.6/mm^2 at 24 h and 15.3 ± 4.3/mm^2 at 48 h) were found in the dermis.

Since it was reported by previous investigators that Thy-1$^+$GA1$^+$ cells are NK cells, we assumed that NK cells infiltrated nonspecifically in the dermis in delayed-type skin reaction by DNFB. In the tumor transplant experiments, the GA1$^+$ cells were found near both types of tumors, but they were in contact with RL♂1 and not with p815. Because it was reported that GA1$^+$ monocytes do not have cytotoxicity against tumor cells, our findings suggest that GA1$^+$ cells migrate nonspecifically to the sites of inflammation, and that the NK cells among them may make direct contact with the tumor cells when they encounter NK cell sensitive tumors.

Key Words: NK CELLS, MOUSE SKIN, IMMUNOHISTOCHEMISTRY

Abbreviations used in this paper:

NK	:	natural killer
DNFB	:	1-fluoro-2,4 dinitrobenzene
GA1	:	asialo GM1 = Ganglio-N-tetraosylceramide
TIC	:	tumor infiltrating cell
HRP	:	horseradish peroxidase
CTL	:	cytotoxic T-lymphocyte
LGL	:	large granular lymphocyte

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INTRODUCTION

Natural killer (NK) cells, a type of cytotoxic lymphoid cell, were reported at first to have natural cytotoxicity against tumor cells in vitro.[1,2,3] These cells were later found to have cytotoxicity against tumor cells in vivo, since NK activity was associated with cells that infiltrated tumors in experimental animals.[4,5,6,7] In human, the presence of many Leu-7$^+$ cells in contact with tumor cells in Hodgkin's lymphoma and liver tumor were noticed.[8] In mycosis fungoides (human cutaneous T-cell lymphoma), Leu-7$^+$ cells were found to constitute 5 % to 10 % of the infiltrating cells.[9] These cells can effectively lyse a variety of tumor cells, virally infected cells, and some micro-organisms, and have been implicated to participate in the body's defense mechanism.[10] But it was difficult to ascertain whether NK cells among tumor infiltrating cells (TIC) play a role in antitumorigenic reaction or whether they are present as a part of a non-specific inflammatory reaction, since many other inflammatory cells like neutrophils, macrophages, and lymphocytes are also seen at the site of tumors.

Our main objective was to explore the mode of NK cell migration to the sites of inflammation and tumor transplantation. Three approaches were used: 1) mice ears were physically irritated or 2) chemically irritated to produce inflammatory lesions, and 3) tumor cells were transplanted to some ears. In the inflammatory lesions and TIC, the NK cells were identified immunohistochemically by their morphology and the presence of GA1 on their surfaces. Since some non-NK cells of peripheral leukocytes are also known to express GA1 at times,[11,12] when possible, immunohistochemical double-staining for GA1 and Thy-1 was also done to assure further identity of the cells, as it has been reported that Thy-1$^+$GA1$^+$ cells are NK cells and that they are distinct from ordinary mature T cells.[13,14] The advantages of using mice ears for these experiments were that ears have often been used for the examination of contact dermatitis, easy to approach, have simple morphology and have little hair.

MATERIALS AND METHODS

Animals: C3H and BALB/c mice were obtained from Central Institute for Experimental Animals, Kawasaki, Japan. Mice were age matched (5 to 8 wks) for each experiment and fed pelleted food and water *ad libitum*.

Antibodies: (a) Primary antibodies – Rabbit anti-GA1 serum was purchased from Wako Chemicals, Osaka, Japan, and monoclonal anti-Thy-1 was purchased from Becton Dickinson, California. (b) Secondary antibodies – Goat anti-rabbit IgG purchased from MBL was labeled with horseradish peroxidase (HRP) by the method of Wilson and Nakane.[15] Peroxidase-labeled goat anti-rat IgG (HRP-G anti-R IgG) was purchased from Tago, Inc., California.

Reagent: 1-Fluoro-2,4 dinitrobenzene (DNFB) was purchased from Kanto Chemical Co., Inc, Tokyo, Japan.

Treatment of animals: To observe inflammation by physical irritation, dry ice was applied to mice ears for 20 sec, and samples were fixed 6 h, 12 h, 24 h, 48 h, 4 days and 7 days after the initial irritation. To observe inflammation by chemical irritation, DNFB was used. For primary irritation, 0.05 ml of 1 % DNFB was applied to the inner side of C3H mice ears and samples were fixed after from 6 h to 4 days. For delayed-type skin reaction, the method according to Phanuphak was used.[16] C3H mice abdomens were shaved and painted with 50 μl of 0.5 % DNFB in 3:1 acetone-olive oil once a day for two consecutive days, and four days after the last painting, challenged on the inner side of the ears with 0.1 % DNFB, and fixed after from 6 hrs to 7 days. For the negative control, only the solvent (acetone and olive oil in the ratio of 3:1) was applied on mice abdomens, and after four days, 0.1 % DNFB was applied on the inner side of the ears. Spleens of untreated adult C3H mice

were used as positive controls. Experimental, positive controls and negative controls consisted of at least six age and sex-matched animals.

Tumor cells: NK sensitive RL♂1 (radiation-induced mouse leukemia in BALB/c mice) cells, and NK cell resistant p815 (mouse mastocytoma in DBA/2 mice) cells were cultured in the Department of Pathology, Tokai Univesity School of Medicine. RL♂1 cells (3×10^8/0.05 ml/ear) were transplanted into C3H and BALB/c mice ears, and p815 cells (3.3×10^6/0.05 ml/ear) were transplanted into C3H mice ears. The ears were fixed after from 6 h to 7 days. As the negative control, C3H mice ears were injected with 0.05 ml of tumor cell medium RPMI + 10 % fetal calf serum (FCS).

Indirect immunoperoxidase method: Samples were fixed in periodate-lysine-paraformaldehyde (PLP) for 6 h and embedded in OCT-compound (Lab-Tek products). These were sectioned at 6 μm thickness in a cryostat and mounted on albumin-coated slides. Endogenous peroxidase in the tissue sections was inactivated by incubation with 0.1 % hydrogen peroxide in PBS and sections were incubated with rabbit anti-GA1 serum overnight at 4°C. Some of the sections were reacted with 10 % non-immune rabbit serum as a control. Subsequently, the sections were incubated in HRP-labeled goat anti-rabbit IgG for 3 h at room temperature with extensive rinsing in between incubations. The reaction products were visualized after incubation with Karnovsky's (DAB-H_2O_2) solution and then mounted.

Double-staining technique: To confirm whether GA1 and Thy-1 antigens were expressed on the same cells or not, two-color immunohistochemistry was done. After DAB-visualization of Thy-1, the slides were washed with PBS in ice and then washed three times in 0.1 M glycine-HC1 buffer at room temperature with constant stirring. After washing with PBS in ice, the slides were reacted with rabbit anti-GA1 serum overnight at 4°C. The slides were washed and incubated with HRP-labeled goat anti-rabbit IgG for 3 h at room temperature. Binding was visualized after incubation with 4-chloro-1-naphthol, mounted in glycerine:PBS (9:1) and photomicrographs were taken immediately.

Semiquantitative analysis: The slides were examined under light microscope at a magnification of x400. By using a calibrated ocular grid, ten contiguous fields/specimen were chosen and reactive cells were counted. Cell numbers were expressed as mean ± SD per mm^2.

RESULTS

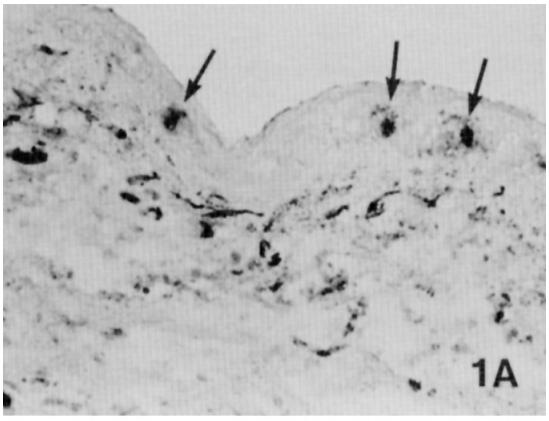
1. NK cells in inflammatory lesions

(a) Normal skin: In C3H mouse ear skin in which no irritant was applied, we found only a few lymphocytes and histiocytes, but no $GA1^+$ cells.

(b) Irritation by dry ice: In C3H mice ears after 6 h of stimulation, the dermis became edematous, and many granulocytes, lymphocytes, histiocytes and a few $GA1^+$ cells were seen infiltrating it. In the epidermis, several $GA1^+$ cells were found (Fig. 1-A). Infiltration of cells was numerous until after four days, and inflammation disappeared after seven days (Table 1). After double-staining with Thy-1, numerous Thy-1^-GA1^+ cells were found in the epidermis, but no Thy-1^+GA1^+ or Thy-1^+GA1^- cells were found (figure not shown). Infiltration of cells was highest after 24 h (108.8 ± 4.5 cells/mm^2), but decreased at 48 h (71.2 ± 3.8 cells/mm^2) as shown in Table 2.

(c) Primary irritation by DNFB: In C3H mice ears, slight vacuolar degeneration was seen in the epidermal cells after 6 h. Degeneration advanced until after four days. In the epidermis, only a few infiltrating granulocytes were found, whereas $GA1^+$ cells and Thy-1^+ cells were not found.

(d) Delayed-type skin reaction by DNFB: In C3H mice ears, no significant inflammation was found within 12 h. After 24 h, lymphocytes, histiocytes, granulocytes and $GA1^+$ cells were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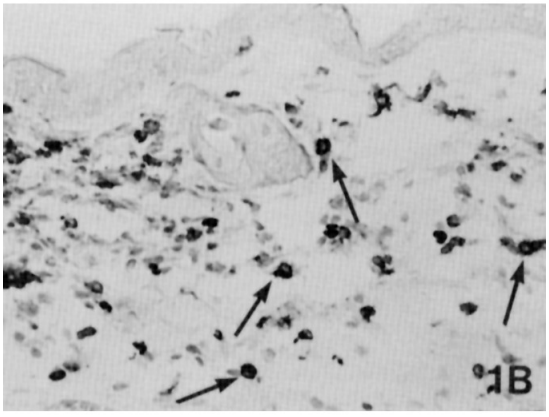
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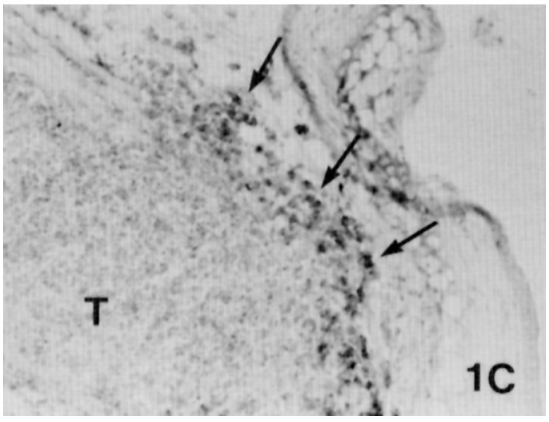
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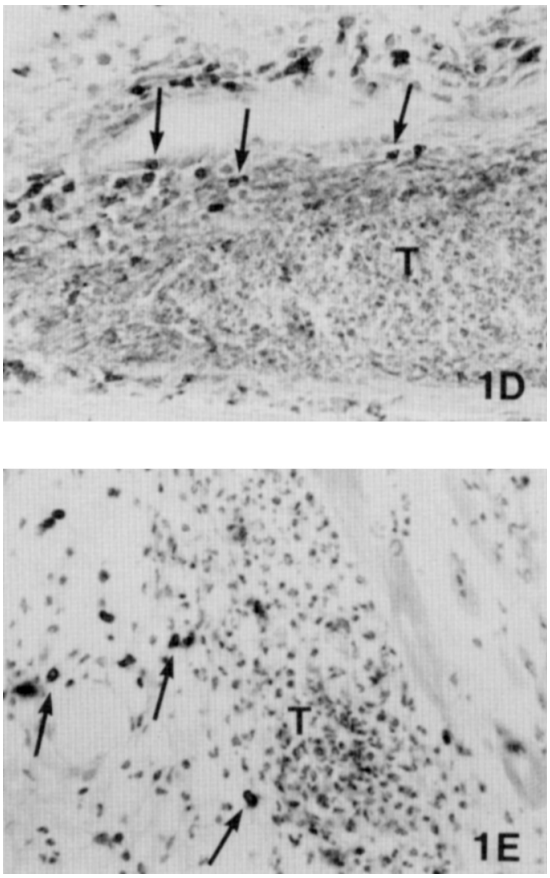

Fig. 1. Indirect immunoperoxidase staining with GA1 of mouse ear skin in different experimental conditions:

A. C3H ear 48 h after freezing with dry ice. $GA1^+$ cells (arrow) were observed in the epidermis. (x320)

B. Delayed-type skin reaction by DNFB: C3H ear 24 h after challenge. $GA1^+$ cells were observed in the dermis. The circumferences of $GA1^+$ cells were stained brown (arrow). Granulocytes were stained brown throughout the cells. (x320)

C. C3H ear 24 h after transplantation of RL♂1. $GA1^+$ cells (arrow) and diffuse GA1 were in contact with the tumor mass (T). (x320)

D. BALB/c ear 48 h after transplantation of RL♂1. $GA1^+$ cells (arrow) and diffuse GA1 were in contact with the tumor mass (T). (x320)

E. C3H ear 24 h after transplantation of p815. $GA1^+$ cells (arrow) were seen around the tumor mass (T). (x320)

Table 1. Surface Densities of AsialoGM1 (GA1) Positive Cells in Different Experimental Conditions.

	6h	12h	24h	48h	4d	7d
Dry ice irritation	+	++	++	++	++	−
DNFB irritation	−	−	++	++	+	−
RL♂1 transplantation in C3H mouse ear	+	+	++	++	+	+
RL♂1 transplantation in BALB/c mouse ear	nd	nd	++	++	++	+
p815 transplantation in C3H mouse ear	+	+	+	+	+	+

− : no cell
\+ : up to 40 cells/mm^2
++ : 40 to 90 cells/mm^2
nd : not done

Table 2. Number of Thy-1$^-$GA1$^+$ Cells and Thy-1$^+$GA1$^+$ Cells in Different Experimental Conditions in C3H Mice.

Type of irritant	Time post-irritation (hr)	No. of cells/mm^2[a]		n[c]
		Thy-1$^-$GA1$^+$	Thy-1$^+$GA1$^+$	
Dry ice	24	108.8 ± 4.5[b]	0	6
	48	71.2 ± 3.8	0	
DNFB	24	87.1 ± 5.8	26.4 ± 3.6	6
	48	60.7 ± 2.9	15.3 ± 4.3	

a. Cells enumerated by calibrated ocular grid of light microscope.
b. Mean ± SD in ten contiguous fields of view.
c. Number of experiments.

infiltrating the dermis (Fig. 1-B). Inflammation was observed until after four days and disappeared after seven days (Table 1). By double-staining, cellular infiltrates were found to be most numerous at 24 h and then declined somewhat at 48 h (Table 2). Thy-1$^-$GA1$^+$ cells, Thy-1$^+$GA1$^-$ cells, and a few Thy-1$^+$GA1$^+$ cells were found to infiltrate the dermis (Fig. 2). We calculated the number of Thy-1$^-$GA1$^+$ cells to be 87.1 ± 5.8/mm^2 at 24 h and 60.7 ± 2.9/mm^2 at 48 h, whereas Thy-1$^+$GA1$^+$ cells were 26.4 ± 3.6/mm^2 at 24 h and 15.3 ± 4.3/mm^2 at 48 h. Infiltration of cells was more marked on the ventral dermis of the ear, the side where chemical stimulation with DNFB was done. There was no change in the epidermal cells, and neither Thy-1$^-$GA1$^+$ cells nor Thy-1$^+$GA1$^+$ cells were found in the epidermis. In the control specimens, histological features were the same as those of normal mouse ear, and both GA1$^+$ cells and Thy-1$^+$ cells were absent.

2. NK cells in transplanted tumors

(a) Transplantation of RL♂1:

In C3H mice ears, a few tumor cells were found in the dermis after 6 h. A tumor mass appeared after 12 h. GA1$^+$ cells were not found at this point. After 24 h, a distinct tumor mass was observed, and GA1$^+$ cells were found around it and were in contact with the tumor cells. Faint, diffuse GA1 also existed around the tumor mass. Inside the tumor mass, neither GA1$^+$ cells nor diffuse GA1 was found (Fig. 1-C). The tumor mass was still observed after seven days. GA1$^+$ cells were also found

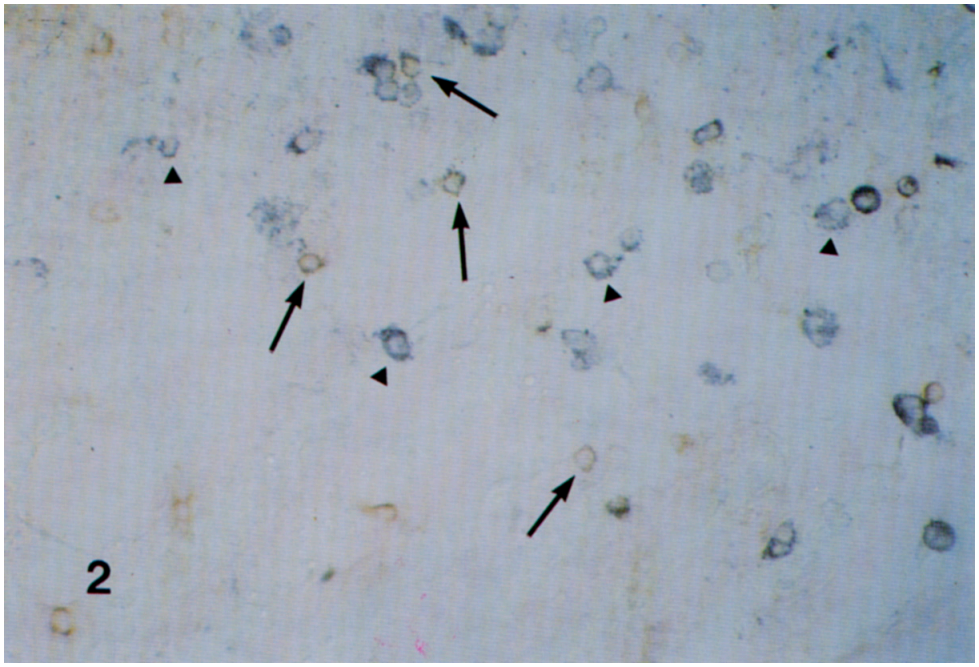

Fig. 2. Immunohistochemical double-staining with GA1 and Thy-1 of C3H mouse ear skin at 24 h post-challenge with 0.1% DNFB: Double-stained brown-blue Thy-1$^+$GA1$^+$ cells (arrow) and blue stained Thy-1$^-$GA1$^+$ cells (triangle) were seen in the dermis. (x400)

after seven days, but they decreased in number gradually (Table 1). In some control sections, inflammatory cells were infiltrating the dermis, and GA1$^+$ cells were found among them.

In BALB/c mice ears, a tumor mass was found in the dermis, and GA1$^+$ cells and diffuse GA1 were observed around it after 24 h. These observations were the same as those of C3H mice (Fig. 1-D).The tumor mass was found to increase gradually until after seven days. GA1$^+$ cells were observed from between one to seven days (Table 1).

(b) Transplantation of p815:

In C3H mice ears, tumor cells were observed after 6 h. Around the tumor mass, inflammatory cells were infiltrating and GA1$^+$ cells were observed among them. But, in contrast with transplantation of RL♂1, GA1$^+$ cells were not in contact with tumor cells, and diffuse GA1$^+$ was not observed (Fig. 1-E). Both the tumor mass and GA1$^+$ cells were still observed after seven days (Table 1).

DISCUSSION

In the present investigation the mode of NK cell migration into inflammatory lesions and areas of tumor transplantation in skin was studied by using mouse as an experimental model. GA1$^+$ cells were absent in normal mouse ear skin and appeared only after physical or chemical irritation. After physical irritation, the GA1$^+$ cells appeared mainly in the irritated epidermis, whereas after

chemical irritation, the cells were found mainly in the irritated dermis. The difference in the location of the $GA1^+$ cells suggests that the cells migrated only into the area of irritation. The epidermis was degenerated after irritation by dry ice and the inflammation mainly occurred in the dermis on the ventral side of ear where DNFB was applied in the delayed-type skin reaction.[16] Those $GA1^+$ cells found in the irritated epidermis were unreactive with anti-Thy-1, but about one fourth of the $GA1^+$ cells in the dermis reacted with the anti-Thy-1.

To isolate and determine NK cell activity of those $GA1^+$ cells with or without Thy-1 found in the area of inflammation is difficult. It was assumed that some of them were NK cells based upon previous reports that Thy-1^+GA1^+ cells are NK cells and that Thy-1^-GA1^+ cells consist of NK cells and some monocytes.[13,14] Most investigators agree that GA1 is present on the NK cells, but all $GA1^+$ cells are not NK cells. Some splenic $GA1^+$ NK cells also express Thy-1 and, by two-color analysis of spleen cells passed through a nylon wool column, it has been shown that a minority of the $GA1^+$ spleen cells were negative for Thy-1.[13] The hepatic large granular lymphocytes (LGL) with NK activity were strongly Thy-1^+,[17] while lung NK-active cells were reported to be Thy-1^-.[18] Thus it appears that depending on where NK cells are found the cells may or may not be accompanied with Thy-1 antigen. There is a possibility that those that migrated to the epidermis and those found in the dermis are slightly different in regard to the expression of Thy-1 antigen. Another possibility for the difference is that the reactibility of $GA1^+$ cells with anti-Thy-1 in the epidermis differs from that in the dermis. Romani et al.[19] were able to demonstrate the Thy-1 antigen only when the epidermal sheets were first reacted with the antibodies and then cryostat sections made. By our method of cryostat section, the fact that the low density of Thy-1 antigen was not revealed maybe another possibility for the difference.

Another area in which NK cells are present is in association with tumors. In our experiments, the migration of $GA1^+$ cells into the areas of tumor transplantation was independent of whether the tumor cells were sensitive to NK cells (RL♂1) or not (p815). However, the pattern of association of $GA1^+$ cells with the transplanted tumors was different. With NK cell sensitive RL♂1, the $GA1^+$ cells were found in juxtaposition to the tumor cells, and GA1 antigen was found diffusely in the area of tumor transplantation. It is possible that the diffusely localized GA1 antigen was released as the $GA1^+$ cells exerted their killing activity upon RL♂1 tumor cells. With NK cell insensitive p815, the $GA1^+$ cells were found in the vicinity of the tumor, but not in direct contact with the tumor cells, and GA1 antigen was confined to the surfaces of the $GA1^+$ cells. It appears that those $GA1^+$ cells in the vicinity of p815 as well as some of those near RL♂1 migrated to the area as the result of non-specific inflammatory reaction, since the injection of an aliquot of medium (RPMI + 10 %FCS) without tumor cells into mice ears also induced the migration of $GA1^+$ cells to the site of injection. As with $GA1^+$ cells found in the areas of inflammation after physical or chemical irritation, there is some uncertainty as to the efficacy of using GA1 antigen as the sole criterion for identification of NK cells in the area of tumor transplantation. In contrast to our co-workers experiment,[14] GA1 antigen was also reported to occur in association with cytotoxic T-lymphocytes (CTL)[20] in addition to NK cells and monocytes/macrophages lineage.[14,21] However, the probability of $GA1^+$ cells that appear near transplanted tumors being CTL is remote. In the experiments using rats, the antitumorigenic reaction of CTL climaxed between four to six days after transplantation of tumor cells, and this immune response was limited to those rats that were immunized with the tumor cells prior to the transplantation.[22] Whereas, in our experiment with the transplanted RL♂1 tumor cells, the $GA1^+$ cells appeared near the tumor as early as 24 h after the transplantation and these mice were not immunized with the tumor cells. In addition, the appearance of $GA1^+$ cells was the same whether RL♂1 tumor cells were transplanted into BALB/c mice or C3H mice. If those $GA1^+$ cells were CTL, more of them should have appeared in the C3H mice, because Major-Histocompatibility Complex (MHC) class 1 antigen of C3H mice is different from that of RL♂1. The possibility that

$GA1^+$ cells may have been of monocyte origin cannot be fully ruled out at this time. However, we feel that the majority of the $GA1^+$ cells that appeared near the transplanted RL♂1 tumor were NK cells, since these $GA1^+$ cells made direct contact with the RL♂1 tumor cells and since monocytes with GA1 antigen have no NK activity.[14]

These observations suggest that NK cells migrate nonspecifically to inflammatory regions as member inflammatory cells, and that when the cells encounter NK cell sensitive tumor cells their killer activity is elicited.

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