MODE OF REGULATION OF NATURAL KILLER CELL ACTIVITY BY INTERFERON*

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Inbred mutant mice that lack a normal T cell system can reject virus-infected tumor cells. For example, as few as 10^2 uninfected HeLa or baby hamster kidney (BHK)[1] cells grow progressively in nude mice, whereas as many as 10^7 virus persistently infected cells fail to grow (1). Spleen cells from nude mice lyse virus-infected but not uninfected HeLa or BHK cells. The characteristics of cells responsible for the in vitro lysis of infected cell lines distinguish them from conventional B cells, T cells, and macrophages, and resemble those ascribed to cells that mediate natural cytotoxic reactions. Subsequent studies (2) indicated that: (*a*) rejection of virus persistently infected tumor cells (virus PI cells) by nude mice was an active process abrogated by irradiation of the mice; (*b*) the effector cells appear to be able to discriminate not only between infected and uninfected tumor cells, but also to distinguish tumor cells persistently infected with different viruses; and (*c*) virus PI cells but not uninfected parental tumor cells inoculated into nude mice in vivo induce significantly augmented in vitro cytotoxicity not only of the homologous target, but also of other irrelevant virus PI tumor cells.

Because of the effectiveness of these cells in mediating cytolysis of virus-infected tumor cells in vitro (2–4) and probably in vivo (5, 6), and the possible role of virus-induced interferon in augmenting this activity (4, 7), we examined the influence of interferon on natural killer (NK)-mediated cytolysis of virus-infected tumor cells. The results indicate that (*a*) Ly 5^+ cells in nude mice mediate all NK activity and (*b*) a subset of these cells produces interferon which, in turn, recruits Ly 5^- precursor cells to acquire surface Ly 5 and NK activity. Thus, virus infection initiates a positive feedback circuit resulting in rapid and efficient amplification of NK function.

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[1] *Abbreviations used in this paper:* AIF, sheep anti-mouse interferon serum; $AsGM_1$, ganglio-*N*-tetraosylceramide; BHK, baby hamster kidney; BSA, bovine serum albumin; C, complement; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; DME, Dulbecco's modified minimal essential medium; EAT, Ehrlich ascites tumor; FCS, fetal calf serum; HeLa-Mps, HeLa-Mumps; HeLa-Ms, HeLa-measles; IF, interferon; MLC, mixed lymphocyte culture, MLTC, mixed lymphocyte-tumor cell culture; NDV, Newcastle disease virus; NK, natural killer; NMS, normal mouse serum; virus PI cell, virus persistently infected cell; VSV, vesicular stomatitis virus.

Materials and Methods

Mice. BALB/c nude mice originally obtained from G. Sato (University of California, San Diego) were maintained in an isolated colony. They were monitored regularly by screens of viruses, and by autopsy of randomly selected mice. CBA/N, CBA/N nude (NIH type II), and C57BL/6 be/be mice, were supplied by Dr. T. Hansen (National Institutes of Health). BALB/c, CBA/J, A/J, C57BL/6, B10.Br., and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). 5- to 8-wk-old mice were used in these experiments, unless specifically mentioned.

Target Cells. HeLa cells uninfected or persistently infected with measles, mumps, or vesicular stomatitis virus (HeLa-Ms, HeLa-Mps, HeLa-VSV) were grown in vitro in Eagle's minimal essential medium supplemented with 7% calf serum as described previously (8).

Spleen Cell Cultures

MIXED LYMPHOCYTE-TUMOR CELL CULTURE (MLTC). 10^5 HeLa or virus PI HeLa cells cultured in 1 ml vol overnight formed confluent monolayers in Linbro 24-well (Flow Laboratories, Inc., Rockville, Md.) culture plates. Medium was then drained, and 10^7 normal mouse spleen cells in 5% fetal calf serum (FCS)-Dulbecco's minimal essential minimum (DME) in 1 ml volume were added to the monolayers of tumor cells and cultured for 24–36 h in 5% CO_2. Culture supernates were collected, centrifuged, and then stored at −70°C until use.

MIXED LYMPHOCYTE CULTURE (MLC). 5×10^7 normal C57BL/6 spleen cells were cultured in modified Click's medium with the same dose of mitomycin C (50 μg/ml)-pretreated CBA/J spleen cells in 10 ml medium for 5 d.

Cytotoxicity Assay

NATURAL CYTOTOXICITY ASSAY. HeLa or virus PI HeLa cells were trypsinized, labeled with ^{51}Cr, washed three times, and seeded into flat-bottom microtiter wells at a density of 10^4 cells/well, and incubated overnight. The plates were washed and 0.2 ml of effector cell dilutions or medium was added and incubated 8–10 h, unless otherwise indicated. Specific ^{51}Cr release was calculated as previously (2).

CYTOTOXIC T LYMPHOCYTE CTL ASSAY. 10^6 of 2–3 d CBA/J concanavalin A (Con A) blast cells were incubated with 200 μCi of $Na_2\ ^{51}Cr\ O_4$ for 1.5 h at 37°C. After four washes, 2×10^4 cells in 0.1 ml vol were seeded into round-bottom microtiter wells. 0.1 ml of various dilutions of primary MLC cells (C57BL/6 anti-CBA) were then added to the target cells and incubated for 6 h. 0.1 ml of supernates were harvested from each well, and then 1% Triton X-100 was added again to each well for the maximal release. The specific ^{51}Cr release was calculated as described previously (2).

Antiserum Treatment. Anti-Ly 5.1 serum was raised by immunizing (STS × E)F_1 mice with ERL D cells as described before (9). Spleen cells at a density of $5–7 \times 10^7$ cells/ml in 2% FCS-DME were incubated with anti-Ly 5.1 serum (final concentration 1:25–1:35) for 30 min at room temperature. The cells were then centrifuged and resuspended to the original concentration in 2% FCS-DME containing rabbit complement (C) at a final dilution of 1:10–1:15 depending on the batches. The cells were incubated for 40 min at 37°C in 5% CO_2, and washed. This treatment killed 30–40% of normal CBA/J spleen cells and no more than 10% of normal BALB/c nude spleen cells.

Monoclonal anti-Thy 1.2 antibody was a gift from Dr. Gefter, Massachusetts Institute of Technology. Monoclonal anti-Qa 5 antibody was kindly supplied by Dr. U. Hämmerling, Memorial-Sloan Kettering Cancer Institute. Because these hybridoma antibodies can be used in very high dilutions ($1:10^2$–$1:10^6$), a one-step treatment was routinely performed. Spleen cells at a density of 5×10^7 cells/ml in 2% FCS-DME were incubated with monoclonal anti-Thy 1.2 (final dilution $1:10^6$) or anti-Qa 5 (final dilution 1:100) and rabbit (C) for 40 min at 37°C in 5% CO_2. 30–35% of normal CBA/J spleen cells were killed by anti-Thy 1.2 plus C treatment, whereas fewer than 5% of normal BALB/c nude spleen cells were killed. Anti-Qa 5 plus C treatment killed about 20–25% of normal C57BL/6 spleen cells.

Rabbit antiganglio-*N*-tetraosylceramide ($AsGM_1$) which has been purified and absorbed with GM_1 was a generous gift from Dr. D. Marcus, Albert Einstein College of Medicine. Goat IgG anti-mouse IgG and IgM was purchased from Meloy Laboratories Inc., Springfield, Va.

Interferon (IF) Preparations and Anti-IF Serum

NEWCASTLE DISEASE VIRUS (NDV)-INDUCED L CELL IF. Mouse L cells in confluent monolayers were adsorbed with UV-inactivated NDV for 3 h at a multiplicity 10:1. The virus was then aspirated and the L cells were cultured in fresh 5% FCS-DME for another 18 h. The culture supernates were collected, centrifuged (30,000 rpm, 2 h), and stored at −70°C.

NDV-INDUCED LEUCOCYTE IF. Normal mouse spleen cells were treated with NH_4Cl to eliminate erythrocytes. The cells were resuspended at a density of 5×10^7 cells/ml and incubated with UV-inactivated NDV at a multiplicity of 2:1 for 3 h. The cells were then washed, resuspended in fresh 5% FCS-DME at a density of 1×10^7, and cultured for another 18 h. The supernates were collected, centrifuged, and stored at −70°C.

PURIFIED MOUSE EHRLICH ASCITES TUMOR (EAT) CELL IF. Mouse IF was produced by challenging EAT cells with NDV, and purified to homogeneity as described previously (10). The purified IF sample had a specific activity of 2×10^9 U/mg protein.

SHEEP ANTI-MOUSE IF SERUM. Partially purified sheep anti-mouse IF serum which had a 1.6×10^{-6} neutralization titer, was a generous gift from Dr. I. Gresser, Institut de Réchèrches Scientifiques sur le Cancer, Villejuif, France.

Results

Genetic Control of NK Activity and IF Production Induced by Virus PI Tumor Cells. Both NK activity and IF production in mice are known to be under genetic control (3, 11). By comparing both activities induced by the same virus persistently infected cell lines, it is possible to explore, at a general level, whether a relationship exists between the genetic capability to produce IF and to effect spontaneous cytolytic activity. In order to examine the ability of various tumor cell lines to stimulate IF production, normal spleen cells from BALB/c and CBA/N nude mice were co-cultured with uninfected HeLa cells, HeLa-Ms, HeLa-Mps, or HeLa-VSV for 24 h. Culture supernates were collected and anti-viral activity was assayed by protection of L-cell monolayers against VSV infection. Spontaneous cytotoxicity of the spleen cells against the same tumors was examined concurrently using the 10 h ^{51}Cr release assay. Results of these experiments are illustrated in Fig. 1 a. HeLa cells persistently infected with either measles, mumps, or VSV stimulated IF production by the spleen cells of normal mice. In contrast, uninfected parental HeLa cells, which were resistant to killing by NK cells, failed to stimulate any detectable interferon. Because the supernates of the virus PI cell cultures alone failed to induce IF, IF induction in this system can be attributed to stimulation by the virus PI tumor cells themselves, rather than released free virus. These results demonstrate that spleen cells of normal nude mice preferentially respond to virus PI tumor cells in vitro by IF production as well as by spontaneous cytotoxicity.

As shown in Fig. 1 b, there were significant differences among murine strains in both NK activity and ability to produce IF against various virus PI tumor cells. The strain distribution of NK activity against HeLa cells persistently infected with three different viruses was essentially the same. Among conventional inbred strains tested, CBA/J and CBA/N mice showed the highest activity against all virus PI cell lines, whereas A/J mice had the least activity. Although the sensitivity of the measurement of IF produced in this system is low, the strain distribution profile of IF production was similar. CBA/J mice produced the highest level of IF, although differences in IF production between intermediate and low NK strains could not be clearly differentiated.

The independence of NK activity and IF production of H-2 control in this model is illustrated in panel 1 b in which congenic mice containing the $H\text{-}2^k$ gene,

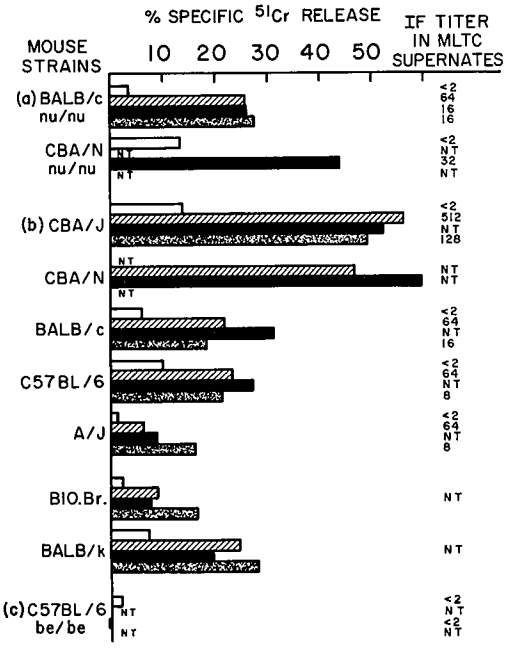

FIG. 1. Strain distribution of NK activity and IF production. Spleen cells from two to three normal mice of each inbred strain were pooled and assayed for NK activity. Spleen cells (10^6) were added to ^{51}Cr-labeled HeLa (□), HeLa-Ms (■), HeLa-Mps (■), or HeLa-VSV (■) cells at L:T ratio 100:1 for 10 h. The means of specific ^{51}Cr release are shown. For the assay of IF production, 10^7 normal spleen cells were co-cultured with 10^5 HeLa-Ms, HeLa-Mps, or HeLa-VSV for 24 h. The culture supernates were then collected, centrifuged, and antiviral activity was assayed as described in Materials and Methods. NT, not tested.

characteristic of CBA and C3H mice, are found to produce no greater degree of NK activity or IF than their parental strains. However, spleen cells from homozygous be/be mice on a C57BL/6 background failed to exert any significant cytotoxic activity or IF production, confirming that the beige mutation prevents the expression of NK activity (12). The strain distribution profile in NK activity on virus PI xenogeneic tumors parallels well that reported on murine lymphoma target cells (3). Taken together these experiments reveal a positive correlation in the genetic control of the ability to generate NK activity and the ability to produce IF upon stimulation with virus PI cell lines.

Phenotype of Lymphocytes Responsible for NK Activity and IF Production. Cells that mediate natural killing are nonadherent and resistant to anti-Ig plus C (2, 3), but are partially sensitive to Thy 1.2 serum plus C (13). Moreover, NK activity of spleen cells from nude mice, which contain fewer than 5% Thy 1^+ cells, is 30–40% reduced by anti-Thy 1 plus C (2). Because NK cells also express the Ly 5 surface alloantigen (14), we examined the effects of both Ly 5.1 antiserum and monoclonal Thy 1.2 antibodies on both NK activity and IF production. Treatment of nude mouse spleen cells with anti-Ly 5.1 plus C eliminated almost all NK activity; whereas treatment with anti-Ly 5.1 serum alone or C alone had no effect (Table I). This depletion is specific because cytotoxic activity of spleen cells from STS/A mice (phenotype Ly 5.2) was unaffected by exposure to anti-Ly 5.1 antibody plus C. IF production by spleen cells from both

TABLE I

Effect of Anti-Thy 1.2, Anti-Ly 5.1, Anti-Qa 5, and Anti-AsGM$_1$ Plus C Treatment on NK Activity and IF Production of Mouse Spleen Cells in Response to HeLa-Ms Cells

Experiment	Mouse strain	Treatment of cells	Specific ^{51}Cr release	IF titer in MLTC supernate
			%	*U*
I	BALB/c, nu/nu (Ly 5.1)	None	37.1 ± 1.4	32
		C alone	35.8 ± 1.1	32
		Anti-Ly 5.1 alone	30.3 ± 1.7	32
		Anti-Ly 5.1 plus C	1.4 ± 0.5	0
		C alone	46.6 ± 1.0	NT
		Anti-Thy 1.2 plus C	31.8 ± 1.8	NT
	STS/A (Ly 5.2)	C alone	23.4 ± 3.7	NT
		Anti-Ly 5.1 plus C	25.1 ± 2.0	NT
II	CBA/J	C alone	53.3 ± 0.6	16
		Anti-Thy 1.2 alone	54.4 ± 1.1	16
		Anti-Thy 1.2 plus C	26.0 ± 2.5	12
		Anti-Ly 5.1 alone	50.4 ± 2.3	16
		Anti-Ly 5.1 plus C	14.3 ± 0.8	0
		Anti-AsGM$_1$ alone	53.8 ± 0.9	8
		Anti-AsGM$_1$ plus C	13.3 ± 2.2	4
III	C57BL/6 (Qa 5$^+$)	C alone	25.4 ± 1.7	16
		Anti-Qa 5 plus C	2.2 ± 2.0	4

* Spleen cells from normal mice were treated with antiserum as described in Materials and Methods, washed, and separated into two parts. One aliquot of the treated cells was added to the ^{51}Cr-labeled HeLa-Ms cells at the 100:1 L:T ratio, and the cytotoxicity was assayed at 10 h. The other portion was co-cultured with HeLa-Ms cells (MLTC) for 24 h. The culture supernates were harvested, centrifuged, and the antiviral activity was assayed as described in Materials and Methods. NT, not tested.

normal and nude inbred mice (phenotype Ly 5.1) was also eliminated after treatment with anti-Ly 5.1 plus C. Taken together, these data demonstrate that both NK activity and IF production were dependent upon Ly 5$^+$ spleen cells,[2] which, in nude mice, represent no more than 7% of the total spleen mononuclear cell population.

Cytotoxic activity of both normal and nude mice spleen cells was partially reduced (~30%), after treatment with monoclonal anti-Thy 1.2 plus C (Table II, experiment I). This finding suggested the possible existence of two NK subsets bearing different phenotypes: Ly 5$^+$ Thy 1$^-$ and Ly 5$^+$ Thy 1$^+$. When spleen cells from nude mice were passed over nylon-wool columns, the nonadherent fraction killed ~50% of HeLa-Ms targets, and the adherent fraction lysed 25% of the targets at an E:T ratio of 100:1. About 50% of NK activity in the nylon-adherent fraction and ~15% of NK activity in the nonadherent fraction of spleen cells was lost after incubation with monoclonal α-Thy 1.2 plus C. NK activity carried by either adherent or nonadherent populations was lost after incubation with anti-Ly 5.1 plus C. Two other surface determinants have been detected on NK cells – AsGM$_1$ (15) and Qa 5 (16). As shown in Table I,

[2] In this paper, the phenotypes Ly 5$^+$ and Qa 5$^+$ represent the susceptibility of cells to lysis by antibody in the presence of complement.

TABLE II

Evidence that the Augmentation of NK Activity by Virus PI Cells Is Mediated through IF

Experiment	Pretreatment of spleen cells*	Specific ^{51}Cr release (% augmentation) on HeLa-Ms spleen cells from:	
		BALB/c, nu/nu	CBA/J
A	Medium	14.4	19.4
	(HeLa plus spleen cells) MLTC supernate‡	14.4 (0)	NT§
	(HeLa-Ms plus spleen cells) MLTC supernate‡	29.5 (105)	28.1 (45)
	(HeLa-Ms plus spleen cells)MLTC supernate plus AIF‖	NT§	17.2 (0)
	HeLa-Ms alone culture supernate	NT§	21.8 (12)
B	Medium	19.7	19.4
	(NDV plus spleen cells) supernate¶	33.2 (69)	48.7 (151)
	(NDV plus spleen cells) supernate plus AIF‖	18.5 (0)	NT§
C	Medium	15.5	19.0
	Purified mouse EAT-IF**	36.2 (134)	28.2 (48)

* BALB/c nu/nu or CBA/J spleen cells were incubated with various samples at 37° C in 5% CO_2 for 0.5–2 h, washed, and then their cytotoxic activities were assayed on HeLa-Ms at 10 h.

‡ CBA/J spleen cells (1×10^7 cells) were co-cultured with 10^5 of HeLa or HeLa-Ms cells for 24 h, and the culture supernates were collected. The IF titers were 0 and 256 U, respectively.

§ NT, not tested.

‖ Sheep anti-mouse IF (AIF) serum. The final concentration was 1:100.

¶ CBA/J spleen cells treated with NH_4Cl were incubated with UV-inactivated NDV at 2:1 multiplicity, washed, and then cultured for 18 h. The supernate was collected, and ultracentrifuged. The IF titer was 512 U.

** Purified mouse EAT-IF (sp act 2×10^9 U/mg protein) was diluted and used at final concentration of 1,500 U.

experiment II, NK activity of CBA spleen cells was drastically reduced by anti-$AsGM_1$ plus C. In experiment III, αQa 5 plus C treatment was found to eliminate completely NK activity of C57BL/6 mice. Of interest, comparable treatment of alloreactive T cells had no effect (U. Hämmerling, Personal communication; Our unpublished data.). These same treatments with anti-Qa 5 and anti-$AsGM_1$ plus C, led to a marked reduction in IF production in response to HeLa-Ms.

These results indicate that natural cytotoxic activity and IF production are mediated by the same set of Ly 5^+, Qa 5^+, $AsGM_1^+$ splenic lymphocytes, and that a subset of NK cells may carry the Thy 1^+ surface marker in addition. It remains to be established whether the same cell engages in IF production and cytotoxicity.

The Ly 5 Alloantigens as a Possible Functional Marker for NK Cells. Recent evidence indicated that Ly 5 antisera (in the absence of C) specifically inhibited NK activity against lymphoma targets (14). As shown in Fig. 2 a, anti-Ly 5.1 serum in the absence of C suppressed the cytotoxicity of HeLa-Ms in a dose-dependent fashion, whereas normal mouse serum (NMS) had only marginal effect. NK activity of normal nude mice against HeLa-Ms was more than 80% suppressed at a final concentration of 1:20 of the antiserum. On the other hand, neither anti-Qa 5 nor anti-$AsGM_1$, which are

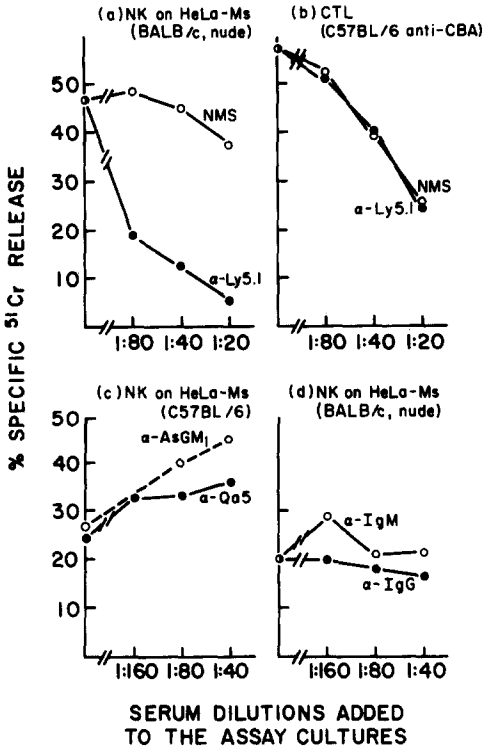

FIG. 2. Effects of the addition of various antisera on NK or CTL activity in the absence of C. Normal spleen cells from appropriate mice were added to ^{51}Cr-labeled HeLa-Ms cells in the presence of various dilutions of NMS or anti-Ly 5.1 serum (a), anti-Qa 5 or anti-AsGM$_1$ (c), or goat anti-IgM or anti-IgG (d), at 100:1 L:T ratio for 10 h. In (b), 5-d primary MLC cells (C57BL/6 anti-CBA/J) were added to ^{51}Cr-labeled CBA-Con A blast cells in the presence of NMS or anti-Ly 5.1 were at 50:1 L:T ratio for 6 h. Each point represents the mean percent specific ^{51}Cr release.

reactive with NK effector cells, affected NK activity when added to the assay culture in the absence of C (Fig. 2 c).

Because CTL also bear Ly 5 at their surface, the effect of anti-Ly 5 serum (in the absence of C) on Ly 5$^+$ T killer cells was also examined (Fig. 2). Ly 5.1 antiserum did not suppress cytolytic activity of alloreactive T cells to any greater extent than did NMS.

Because anti-Qa 5 and anti-AsGM$_1$ as well as anti-mouse Ig antibodies (Fig. 2 d) in the absence of C failed to block NK activity, it is unlikely that the anti-Ly 5 serum blocks nonspecifically merely by competing for Fc receptors on NK cells and by interfering with target cell binding. These results suggest that the Ly 5 gene product is critically involved in the recognition and/or cytolytic process of NK cells.

IF Is Responsible for Augmented NK Activity by Virus-infected Tumor Cells. After inoculation of nude mice with virus PI tumor cells, spleen cells exert significantly greater natural cytotoxic activity in vitro (2). Because (*a*) virus-infected cell lines can induce spleen cells to produce IF and because (*b*) increased IF levels have been associated with enhanced NK activity, we tested the possibility that supernates containing IF might augment NK activity in vitro. Incubation of spleen cells for 3 h with supernates of

MLTC resulted in a substantial increase in subsequent lysis of HeLa-Ms targets. This augmentation is dependent on IF in the supernate because (*a*) incubation of spleen cells with supernates of cultures of uninfected HeLa cells and spleen cells, or with supernates of Ms virus persistently infected HeLa cells alone, lack IF activity and produce no NK augmentation (Table II A); (*b*) augmentation by MLTC supernates is lost after addition of sheep anti-mouse IF Ig (Table II A) to the supernate; and (*c*) NK activity was also augmented by NDV-induced mouse leukocyte IF (Table II B). In these, as well as all previously published experiments it could be argued that the NK-augmenting activity was, in fact, not IF, but a contaminant. To demonstrate formally that IF, and not contaminants, were involved, we were able to use chemically homogeneous purified mouse IF of sp act 2×10^9 U/mg (Table II C). The augmentation by purified IF directly demonstrates that it is IF itself which exerts the major immunoregulatory effect on natural cytotoxic activity. Taken together, these data indicate that IF augments natural cytotoxic activity, and that IF is produced by Ly 5^+ spleen lymphocytes after incubation with virus-infected cells.

This result raised the possibility that apparent NK activity against virus-infected cells might be enhanced by IF produced by Ly 5^+ cells during incubation with virus-infected targets. We therefore examined the kinetics of lysis of virus-infected target cells in the presence and absence of anti-IF antibodies (capable of neutralizing greater than 10^3 IF units). Anti-IF serum did not significantly reduce lysis in the early phase of the assay, i.e., less than 12 h (Fig. 3 a). However, anti-IF serum significantly reduced lysis during the later phase of the assay. In this early phase, the IF-independent, effector activity is still sensitive to α-Ly 5 plus C, indicating that preexistent NK effector cells express Ly 5 alloantigen, eliminating the possibility that the sole role of the Ly 5^+ cell would be to produce IF required by another cell type which might effect killing. The results indicate that lysis observed early in this NK

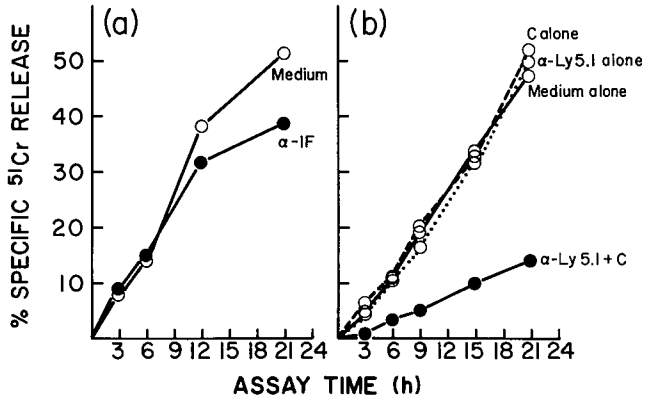

FIG. 3. Spontaneous and IF-dependent cytotoxicity in the NK assay system. (a) Normal CBA/J spleen cells were added to the ^{51}Cr-labeled HeLa-Ms cells at A:T ratio 100:1. At the same time, rabbit anti-IF IgG (AIF) was also added to the assay culture at the final concentration of 1:200. 3, 6, 12, and 21 h later, the culture supernates were harvested and percent specific ^{51}Cr release was determined as described in Materials and Methods. ○, no AIF added; ●, AIF added. (b) Normal CBA/J spleen cells were treated first with medium (○——○), C alone (○- - -○), anti-Ly 5.1 alone (○-·-○), or with anti-Ly 5.1 plus C (●——●). After two washes, the treated cells were then added to the ^{51}Cr-labeled HeLa-Ms cells at A:T ratio 100:1. 3, 6, 12, and 21 h later the culture supernates were harvested, and the percent specific ^{51}Cr-release was determined. Each point represents the mean of triplicate culture.

assay (< 10 h) is independent of IF production in the assay culture and represents a reliable assay of preexisting NK activity. These observations indicate that it is necessary to determine the conditions that allow direct measurement of NK function in the absence of IF augmentation in assay cultures.

Mode of Augmentation of NK Activity by IF. All NK cells and IF-producing cells stimulated by virus-infected tumor cells are Ly 5^+ and Qa 5^+. In order to pursue the mechanism by which IF exerts its immunoregulatory influence, it was necessary to define the precise cell upon which IF acts. Two possibilities are apparent: IF acts on Ly 5^+ Qa 5^+ NK cells and activates them or enhances their cytolytic activity; and IF acts on a precursor to NK cells promoting its differentiation to an effector cell. In order to discriminate between these possibilities we first determined whether IF acted on Ly 5^+ or Ly 5^- cells. Spleen cells from BALB/c nu/nu or CBA/J mice were treated with C alone or with anti-Ly 5 plus C. After washing, each group of cells was divided into three portions and incubated for 3 h at 37°C with medium alone, with NDV-induced mouse leukocyte IF, or with culture supernates containing IF, before assessment of NK activity against virus-infected HeLa cells in a 10 h assay. NK activity of control (C-treated) nude mouse spleen cells was augmented after preincubation with IF or with MLTC supernates. Spleen cells treated with anti-Ly 5 plus C expressed very low cytotoxic activity, confirming that NK activity requires Ly 5^+ cells. However, spleen cells pretreated with anti-Ly 5 plus C before exposure to IF or MLTC supernates, showed markedly augmented NK activity (Fig. 4). The degree of augmentation of Ly 5^- cells by IF was comparable with or greater than control populations containing Ly 5^+ cells. The same type of experiment was performed using anti-Qa 5 antibody. As shown in Fig. 4 c, C57BL/6 spleen cells pretreated with C alone showed significantly augmented NK activity after incubation with NDV-induced leukocyte IF (5–19%). However, no augmentation of the cytotoxic activity was observed in the anti-Qa 5 plus C pretreated group following the incubation with

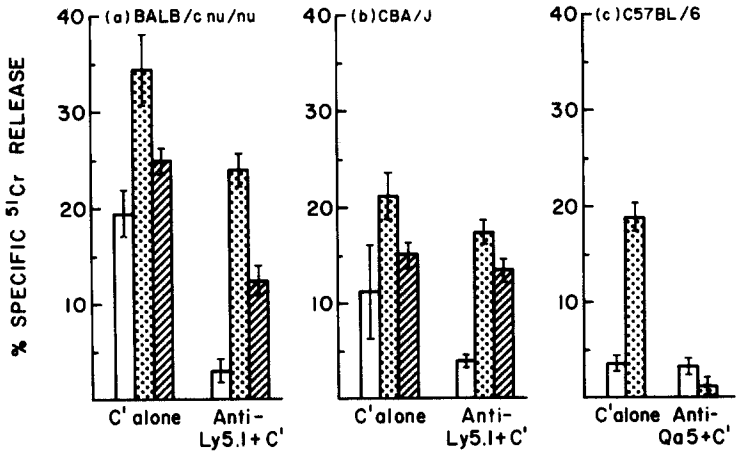

FIG. 4. Normal spleen cells from 7-wk-old BALB/c nude mice (a), 7-wk-old CBA/J mice (b), or 6-wk-old C57BL/c mice (c) were first treated with C alone, anti-Ly 5.1 plus C or with anti-Qa 5 plus C. After washing, each group of spleen cells was incubated with medium (□), 500 U of NDV-induced mouse leukocyte IF (▩), or with 250 U of HeLa-Ms-induced mouse IF (▨), for 3 h at 37°C in 5% CO_2 incubator. The cells were then washed and added to the ^{51}Cr-labeled HeLa-Ms cells for 10 h. The mean percent specific ^{51}Cr release of triplicate cultures ± SE is shown.

IF (4–2%). These results indicate that Ly 5^+, Qa 5^+ cells are required for NK activity and production of IF, and that the augmentation of NK activity by IF is accounted for by its effect on noncytotoxic Ly 5^- Qa 5^+ cells. In sum, these data support the second of the two hypotheses above, namely that IF acts on Ly 5^- Qa 5^+ precursor of NK cells.

To establish whether the Ly 5^-, Qa 5^+ cell acted upon by IF is a precursor to the functional NK cell, we asked whether NK activity resulting from exposure of Ly 5^- nude mouse spleen cells to IF was mediated by Ly 5^+ cells (Table III). NK activity of Ly 5^- cells incubated 3 h with IF was lost if the cells were subsequently treated with anti-Ly 5 plus C after IF induction. These results demonstrate that IF can induce Ly 5^-, Qa 5^+ precursors to express both NK activity and the associated Ly 5 surface component.

That IF can act on a cell which is different from the NK effector cell was confirmed in independent experiments in which the augmentation of cytotoxicity induced by IF was assessed in spleen cell fractions obtained from bovine serum albumin (BSA) gradient fractionation (17). Whereas IF augmented the cytotoxic activity of the densest fraction of the spleen cells to the greatest extent, the greatest preexistent cytotoxic activity was found in the least dense fraction. Although these populations are hardly pure, the results confirm that IF acts on a fraction of cells not possessing NK activity.

Discussion

An intimate relationship between NK activity and IF production was confirmed in these studies. Comparisons between inbred and congenic strains demonstrated a striking parallel between NK activity and IF production, largely independent of the H-2 locus. Because the strain distribution of NK activity on virus PI cell lines parallelled that found on lymphoma targets (YAC), which failed to show cross-competition with any of the virus-infected targets studied, the results suggested that the genetic influence on NK activity may either be expressed in the lytic mechanism of the effector cells, or more likely, on IF production itself, rather than on the target cell recognition process. It is noteworthy that whereas the strain distribution profile

TABLE III

Induction of Ly 5.1$^+$ NK Effector Cells by IF from Ly 5.1$^+$ Cell-depleted Spleen Cells

Pretreatment of the spleen cells			Specific ^{51}Cr release	
Step 1	Step 2	Step 3	Experiment I	Experiment II
			%	
Medium	Medium	Medium	20.5	14.2
Medium	IF	Medium	32.8	23.0
Anti-Ly 5.1 plus C	Medium	Medium	7.2	4.6
Medium	Medium	Anti-Ly 5.1 plus C	3.7	—
Anti-Ly 5.1 plus C	IF	Medium	22.0	14.7
Anti-Ly 5.1 plus C	IF	Anti-Ly 5.1 plus C	3.7	0.0

* Normal nude mouse spleen cells treated with medium or anti-Ly 5.1 plus C (step 1) were preincubated with medium or purified mouse IF (1,500 U) for 1–2 h at 37°C in 5% CO_2 (step 2), washed, and again treated with medium or anti-Ly 5.1 plus C (step 3). The NK activity of the cells was then asayed on HeLa-Ms cells (experiment I) or on HeLa-Mps cells (experiment II) at L/T ratio 100:1 at 10 h.

of IF production induced by virus PI tumor cells in vitro closely followed that of NK activity, there were significant differences in the patterns of IF production induced by the virus PI cell lines in vitro compared with those reported for IF induction in vivo by free viruses (11).

Trinchieri and Santoli (4) made the fundamental observation that NK effector cells and IF-producing cells belong to the same fraction of human peripheral blood lymphocytes. The studies reported here demonstrate that in conventional and nude mice, both IF-producing and NK activities are mediated by a subset of lymphocytes, representing less than 5% of the total mononuclear cells that express the Ly 5, Qa 5, and $AsGM_1$ surface markers. Ly 5 alloantigens are rather generally distributed among murine lymphocytes, although apparently only T cells and NK cells are sensitive to C-mediated cytolysis (18). $AsGM_1$ antigens have been reported on NK cells and some T cells, but appear to be absent on CTL (15). The expression of the Qa 5 antigen is limited to a subpopulation of peripheral T cells and not present on thymocytes, but found on some non-B/non-T lymphocytes, including NK cells (19). Our data indicate that essentially all NK cells and IF-producing cells express Ly 5, Qa 5, and $AsGM_1$ surface antigens. In addition, a significant portion (35–40%) of the NK cells even of nude mouse spleen cells express the Thy 1.2 antigen. Of some interest was the finding that antisera to surface Ly 5, in contrast with antibodies of the other NK cell markers, specifically blocked NK activity in the absence of complement, without affecting the killing of allogeneic cells by CTL (Fig. 2), suggesting that an Ly 5-associated structure on NK cells may be critically involved in the effector function of these cells.

The studies reported here indicate that after stimulation of spleen cells with virus or virus PI cell lines, IF-containing supernates augment NK activity in vitro, as has been reported in a number of other systems (4, 7). Using chemically homogeneous mouse IF (sp act 2×10^9 U/mg), we have been able to provide unambiguous evidence that the IF molecule itself, rather than a contaminant, is responsible for augmentation of NK activity (Table II). The following two models for the regulation of NK activity by IF were considered: IF potentiates the cytolytic activity of mature NK cells, analogous to the effect of lymphokines on macrophages; and, IF acts on a precursor cell population to differentiate and acquire new (NK) biological function. The results presented here clearly indicate that IF can induce the differentiation of Ly 5^- precursor cells to Ly 5^+ NK effector cells (Table III). In contrast, no induction of NK activity by IF was observed in spleen cells depleted of NK activity by anti-Qa 5 plus C treatment. From this and from the finding that anti-Thy 1.2 pretreatment failed to affect induction of NK activity by IF (data not shown), we can infer that the IF-sensitive population is a Thy 1^-, Ly 5^-, Qa 5^+ cell. The cytotoxic activity generated in a period of 1–3 h exposure to IF of Ly 5-depleted spleen cells was subsequently completely eliminated by retreatment with anti-Ly 5 plus C, demonstrating that the IF-induced effector cell now had the same phenotype as preexisting NK effector cells, namely Thy $1^\pm$, Ly 5^+, Qa 5^+. These results were consistent with results of independent experiments showing that NK cells and the cells acted upon by IF could be partially separated on BSA density gradients (17). Recently, elegant studies on human cells showed that the human NK cell and the IF-responsive cell were distinct subpopulations physically separable on Ficoll gradients (20). Further, the cells binding to targets that appear to be responsible for natural cytotoxicity have been shown by immunofluorescence to produce IF (20, 21) (A. Neighbour. Personal communication.).

A fundamental question raised by these experiments is the relationship between the NK-IF system to other cytotoxic systems. In addition to major histocompatibility complex-restricted T cells, macrophages (22), and promonocytes (23) are reported to be cytotoxic to various tumor cells under appropriate conditions. By morphological (23) and limited serological criteria (23) cells thought to be NK cells bear resemblance to promonocytes. Data of others and our own (2, 14) indicated that NK cells are distinct from conventional macrophages. In this regard, we have recently observed that colony-stimulating factor-sensitive cells, namely promonocytes, are resistant to both anti-Qa 5 and anti-Ly 5 plus C treatment, both of which effectively eliminate NK activity (N. Minato, R. Stanley, and B. R. Bloom. Manuscript in preparation.). Because NK cells share several surface markers in the mouse in common with some T cell populations, i.e., Ly 5, Qa 5, $AsGM_1$, and to some extent Thy 1, the possibility arises that NK cells may be related to the T cell differentiation pathway. Whether Ly 5^+, Thy 1.2^- NK cells differentiate into Ly 5^+, Thy 1^+ NK cells, and whether these cells could further differentiate, under appropriate environmental influences, into mature CTL remain intriguing questions. If it could be established definitively that NK cells have antigen-specific recognition, one could further explore the possibility that NK cells may be T cell precursors which possess an antigen-specific receptor which has not been histocompatibility restricted because it has not been processed by the thymus. In such a model, NK cells would represent an extra thymic alternative pathway to development of specific cytotoxic lymphocytes in the periphery. Such a model raises a number of fundamental problems, including: (*a*) the failure to detect Lyt surface alloantigens on NK cells (24) (N. Minato and B. R. Bloom. Unpublished observations.); (*b*) the fact that in beige mice NK activity is markedly defective although CTL and promonocyte development appear to be normal (25); and (*c*) a theoretical incompatibility with the generally accepted view that the role of the thymus is selective rather than instructive. It is obvious that relating either morphology or surface antigenic determinants to cell lineage is a tenuous extrapolation, and it may well be that NK cells represent either a unique and independent pathway to an effector cell that differentiates no further, or more likely, that there may be indeed more than one type of NK cells, and that the differences in specificity and surface markers reported in different systems represent the functions of different cell types.

There is increasing evidence that NK cells contribute to resistance to tumors and viral infections (5, 6, 26). Several features of this resistance are, we believe, particularly relevant to their action. NK cells show selective cytotoxicity; transformed cells and virus-infected tumor cells are susceptible to killing, whereas primary tumor cells or virus-negative cells are frequently not (2). IF and agents that induce IF augment NK activity in vivo and in vitro. The potential physiological importance of the IF-NK amplifier circuit is indicated by preliminary observations on the treatment of nude mice, which ordinarily reject the virus PI tumor cell lines, with AIF sera (27). Such anti-IF treatment permits growth and metastasis of the virus PI tumor cell lines in vivo. Because the tumor lines studied are of human and hamster origin, and IF is species specific, loss of tumor resistance in vivo with anti-IF treatment most likely reflects the effect of IF on differentiation of NK effector cells, rather than a direct growth inhibitory effect on the tumor cells. We believe that this model may be useful to assess the contributions of the IF-NK circuit to resistance to tumors and viruses.

Summary

Whereas xenogeneic tumors such as baby hamster kidney or HeLa cells grow in nude mice, the same cells persistently infected with a variety of viruses are rejected. Spleen cells from normal nude mice were found to be induced to produce interferon and to exert natural killer (NK) activity on virus persistently infected (PI) tumor cells, and not on uninfected parental cells in vitro. The phenotype of the interferon-producing cells and the NK effector cells was found to be the same namely, Qa 5^+, Ly 5^+, ganglio-*N*-tetraosylceramide, with 35% of the NK cells also expressing Thy 1.2.

NK activity against virus PI tumor cell lines could be nonspecifically augmented both in vivo and in vitro by prior contact with virus PI tumor cells. It was unambiguously demonstrated with chemically homogeneous mouse interferon that interferon, and not a contaminant, was responsible for the augmentation of NK activity in vitro. Studies on the mode of interferon action in augmenting NK activity revealed that the target cell for interferon action was serologically distinct from the NK effector cell. Anti-Ly 5 + complement (C)-treated spleen cells were depleted of NK activity and the ability to produce interferon, but, upon incubation with interferon for 1–3 h, regained both NK activity and susceptibility to anti-Ly 5 + C. Treatment with anti-Qa 5 + C eliminated NK activity, which could not be restored by the addition of interferon. We conclude that interferon produced by Ly 5^+ cells in response to virus PI tumor cells acts on Ly 5^- precursor cells and induces their differentiation into functional Ly 5^+ NK effector cells.

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