ON THE HETEROGENEITY OF MURINE NATURAL KILLER CELLS*

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In the mouse, a number of serological markers are reported to be expressed on natural killer (NK)[1] cells, including NK_1 (1, 2), Ly-5 (3), Qa-2, Qa-4, and Qa-5 (4, 5), asialo GM_1 (6), Thy-1 (7), and Mph-1 (8). The conflicting evidence on the phenotypes and characteristics of NK cells in various systems can best be reconciled if NK cells are not a single cell type derived from a common lineage, but represent a heterogeneous population of cells sharing the capability of selective cytotoxicity. The possibility that NK cells were heterogeneous was initially suggested by the important experiments of Stutman et al. (9, 10) and Burton (2), which indicated that spontaneous cytotoxic effector cells active on solid tumor cells have characteristics different from the NK cells that kill lymphoma cell targets, e.g., cell surface markers, organ distribution, strain distribution, and radiation sensitivity. Kumar et al. (11) observed that mouse NK activity on EL4 target cells had different sensitivity to bone marrow irradiation by ^{89}Sr from that on YAC tumor cells. Lohman-Matthes et al. (8) reported that cultured bone marrow cells could exert NK-like activity, and that such cells shared common surface antigenic determinants with mononuclear phagocytes.

In previous work (12), we have attempted to define some of the genetically determined serological markers on mouse NK cells effective against virus persistently infected xenogeneic tumor cells and, using such markers, were able to show that interferon acts on a precursor cell stimulating its differentiation into a cytolytic effector NK cell. In the present report we have analyzed the heterogeneity of NK cells in mice and identified at least four phenotypically distinct cytotoxic effector cells that differ not only in their surface markers, but also in their responsiveness to the regulatory influences of interferon (IFN) and interleukin 2 (IL-2).

Materials and Methods

Mice. C57BL/6 mice (B/6) were purchased from The Jackson Laboratory (Bar Harbor, Maine). C57BL/6 *be/be* and *nu/nu*, and CBA/N *nu/nu* mice were originally supplied by Dr. K. Hansen (National Institutes of Health), and bred in our own colonies. Throughout the experiments, 6–8-wk-old mice were used.

Tumor Cells. HeLa cells persistently infected with measles virus (HeLa-Ms), the character-

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[1] *Abbreviations used in this paper:* C, complement; CTL, cytolytic T lymphocytes; FCS, fetal calf serum; IL-2, interleukin 2; HeLa-Ms, HeLa cells persistently infected with measles virus; IFN, interferon; MLC, mixed lymphocyte culture; NC, natural cytotoxic; NK, natural killer; NMS, normal mouse serum; TK, T killer.

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istics of which have been described elsewhere (13), were grown in Dulbecco's modified minimal essential medium supplemented with 8% calf serum. P815 cells were grown in RPMI 1640 with 10% fetal calf serum.

Cell Cultures. Single cell suspensions of various mouse lymphoid organs including thymus, spleen, and bone marrow were cultured in RPMI 1640 medium supplemented with streptomycin, penicillin, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum (FCS) in 7% CO_2 in air. The cell concentration was 1×10^7 cells/ml for thymus and spleen cells, and 5×10^6 cell/ml for bone marrow cells.

Antiserum Treatment. Monoclonal anti-Thy-1.2 ascites (New England Nuclear, Boston, Mass.) was used at a final dilution of 5×10^{-3}, and monoclonal anti-Qa-5 ascites (a generous gift from Dr. U. Hammerling, Sloan-Kettering Institute, New York) were used at a final dilution of $1{:}10^{-2}$; anti-Lyt-1.2 serum, anti-Lyt-2.2 monoclonal antibody, and anti-Ly-5.1 serum (kindly supplied by Dr. H. Cantor, Sidney Farber Cancer Center, Boston, Mass.) were used at 1:20, 1:60, and 1:40 final dilutions, respectively. The cells at densities of 5×10^6–1×10^7/0.5 ml were treated either in single step with anti-Thy-1.2 or anti-Qa-5 and rabbit complement (final dilution 1:10) for 35 min at 37°C, or in a two-step procedure with anti-Lyt-1.2, anti-Lyt-2.2, or anti-Lyt-2.2, or anti-Ly-5.1 (30 min at room temperature) and rabbit complement 1:10 for 35 min at 37°C with frequent dispersal.

Cytotoxicity Assay. The nonadherent population of the cultured cells was harvested, washed once, and the cytotoxic activity was assayed using a 6–8-h ^{51}Cr release assay as described previously (13). Spontaneous ^{51}Cr release from HeLa-Ms cells was 10–15%, and 10–20% from P815 cells.

Interferon and T Cell Growth Factor Preparations

IFN. 10^7 cells/ml of normal CBA/J spleen cells were co-cultured with monolayers of HeLa-Ms cells in RPMI 1640-0.5% FCS medium for 24 h. The culture supernates were centrifuged to remove cells, concentrated 50 times by ammonium sulfate precipitation (40–85% saturation), and, after dialysis against three 500 vol of 50 mM potassium phosphate buffer, pH 6.0, the sample was applied to a Bio-Gel P100 column (200 ml volume). Each fraction was assayed for IFN activity, and IFN-positive fractions were pooled and concentrated by ammonium sulfate to a specific activity of $\sim 10^5$ U/mg protein. The characteristics of HeLa-Ms-induced mouse IFN were the following: (*a*) molecular weight ~40,000, with a minor peak at 20,000; (*b*) completely resistant to pH 2.0 treatment for 24 h; (*c*) neutralized by anti-mouse IFN serum (generously supplied by Dr. Ion Gressor, Centre National de la Recherches Scientifique, Villejuif, France).

INTERLEUKIN 2 (IL-2). 10^7 cells/ml of B/6 and BALB/c +/+ or BALB/c *nu/nu* spleen cells were co-cultured (mixed lymphocyte culture) (MLC) in RPMI 1640-0.5% normal mouse serum (NMS) medium for 30–48 h. The culture supernates were pooled and concentrated by ammonium sulfate precipitation (50–100% saturation). The precipitate was resuspended in 20 mM Tris, 0.15 M NaCl, pH 7.2, dialyzed against the same buffer, and applied to a Bio-Gel P100 column (200 ml volume). Each fraction was assayed for both IFN activity and cytotoxicity-enhancing activity, the latter by culturing normal spleen cells with 5–10% vol:vol of each fraction for 2 d and examining the cytotoxicity of the cells on HeLa-Ms cells. As shown in Fig. 1, cytotoxicity-enhancing activity was observed in two peaks. The first peak (45,000 mol wt) corresponded with the peak of IFN activity, whereas the second peak (35,000 mol wt) was free of IFN activity. Preliminary experiments showed that production of both factors was dependent on the Thy-1^+, Lyt-1^+, Lyt-2^-, Qa-5^- cell population, indicating both IFN and the cytotoxicity-enhancing activity used in these experiments derived from T cells. The second peak will be referred to as IFN-free IL-2, and was used at a concentration of 5–10% vol:vol.

Results

Heterogeneity of the Phenotype of Spontaneous Killer Cells Generated in Cultures of Lymphoid Organs of Normal Mice. Cells from mouse spleen, bone marrow, and thymus of C57BL/6 +/+ (B/6), *nu/nu*, and *be/be* mice were cultured in medium for 4–5 d, and the cytotoxic activity of the nonadherent populations was assayed against two different

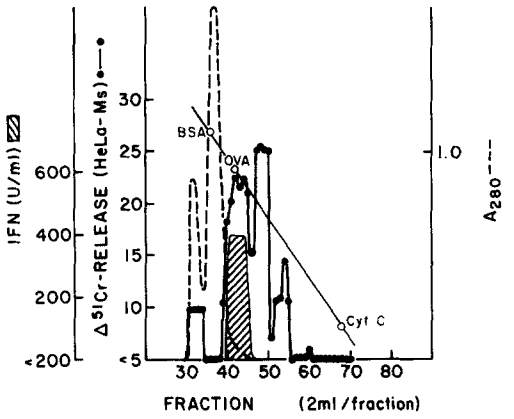

FIG. 1. Separation of IL-2 from IFN activity in MLC supernates. MLC supernates (48 h) were concentrated 50 times and applied to a Bio-Gel P100 column as described in Materials and Methods. Each fraction was assayed for IFN (antiviral) activity (shaded area) and NK-enhancing activity (●). The latter was expressed as the increase in percent specific ^{51}Cr release on HeLa-Ms cells (percent specific ^{51}Cr release by the spleen cells cultured 2 d with 10% vol:vol of each column fraction/percent specific ^{51}Cr release by the spleen cells cultured 2 d with 10% vol:vol column buffer). (----), A_{280} of each fraction.

target cells, namely HeLa cells persistently infected with measles virus (HeLa-Ms), an NK-sensitive target similar to YAC-1, and P815 mastocytoma cells, an NK-resistant tumor cell. To clarify the serologic phenotypes of the effector cells on each target, the cultured cells were treated with various antisera in the presence of complement, including anti-Thy-1.2, anti-Qa-5, anti-Ly-2.2, and anti-Ly-5.1 just before the assay for cytotoxicity. The results of the selective depletion experiments are summarized in Table I. It is clear that at least four serologically distinct cytotoxic populations exist or can be generated in cultures of normal B/6 lymphoid organs. They are the following: (*a*) Thy-1$^+$, Qa-5$^-$, Lyt-2$^+$, Ly-5$^+$ cells generated from spleen, but not from bone marrow, which are cytotoxic to allogeneic P815 targets and ineffective against HeLa-Ms cells (Table I, A2, B1); (*b*) Thy-1$^-$, Qa-5$^+$, Ly-5$^+$ cells generated from spleen and cytotoxic only to HeLa-Ms (or YAC cells), but not P815 targets (Table I, AI, 2); (*c*) Thy-1$^+$, Qa-5$^+$, Ly-5$^+$ cells, also cytotoxic for HeLa-Ms or YAC-1 but not P815 (Table I, A1, 2); and (*d*) Thy$^-$, Qa-5$^-$, Lyt-2$^-$, Ly-5$^+$ cells found preferentially in cultures of bone marrow and effective only against HeLa-Ms (or YAC) target cells (Table I, B1). No spontaneous killer cells were generated under these culture conditions from thymus cells. It is noteworthy that all NK subsets expressed the Ly-5 surface antigen.

The first type of NK cell (Thy-1$^+$, Qa-5$^-$, Ly-2$^+$) could not be generated in cultures of B/6 *nu/nu* cells (Table I, A3). Because the serological phenotype of this population corresponds to that of conventional alloreactive T killer cells, it will be designated here as the T killer (TK) subset. The second type of cytotoxic cell (Thy-1$^-$, Qa-5$^+$, Lyt-2$^-$) exists in primary spleen cells, but was not found in either primary or cultured B/6 *be/be* spleen cells (Table I, A4). These characteristics, as well as the serological phenotype, correspond most closely to those of the NK cell described in most of the systems reported, and will be referred to here as the NK_I subset, because it is regulated by IFN, as will be described later. The third type of cells representing 30–40% of the NK activity in spleens of normal or nude mice was similarly Qa-5$^+$ and Ly-5$^+$, but

TABLE I

Heterogeneity of the Serological Phenotypes of Spontaneous Killer Cells Generated in the Cultures of Mouse Lymphoid Organs

Effector source	Target cells	Cytotoxicity	Percent reduction of cytotoxicity			
			Anti-Thy-1.2	Anti-Qa-5	Anti-Lyt-2.2	Anti-Ly-5.1 (plus C)
		%				
A. Spleen						
1. B/6 +/+	HeLa-Ms	20	35	100	0	84
fresh	P815	0	—*	—	—	—
2. B/6 +/+	HeLa-Ms	17	75	75	0	70
4-d culture	P815	23	100	40	80	94
3. B/6 *nu/nu*	HeLa-Ms	43	80	65	0	70
4-d culture	P815	0	—	—	—	—
4. B/6 *be/be*	HeLa-Ms	0	—	—	—	—
fresh						
B/6 *be/be*	HeLa-MS	0	—	—	—	—
4-d culture						
B. Bone marrow						
1. B/6 +/+	HeLa-Ms	20	0	10	0	100
4-d culture	P815	0	—	—	—	—
2. B/6 *nu/nu*	HeLa-Ms	28	55	55	0	—
4-d culture	P815	0	—	—	—	—
3. B/6 *be/be*	HeLa-Ms	25	0	0	0	100
4-d culture						
C. Thymus						
B/6 +/+	HeLa-Ms	0	—	—	—	—
4-d culture	P815	0	—	—	—	—

Spleen, bone marrow, or thymus cells from 6–8-wk-old mice were cultured in RPMI 1640-10% FCS medium for 4 d. Fresh or cultured cells were treated with C alone or various antisera plus C, and the cytotoxicity was assayed on HeLa-Ms or P815 cells using the 6-h ^{51}Cr release assay at a lymphocyte to target ratio of 100:1. Assays were performed in triplicate and each value represents the mean of two to three independent experiments.

* Not applicable.

also expressed the Thy-1.2 antigen and is designated the NK_T subset. The fourth type of NK cell, expressing only the Ly-5 marker, was found primarily in cultured bone marrow cells. In contrast to NK_I and NK_T cells, these effector cells were generated equally well in cultures of bone marrow cells from B/6 +/+, *nu/nu*, or *be/be* mice. Because these cells are clearly distinct from the other subsets, they will be designated NK_M cells, because their presence was restricted to bone marrow. As shown in Table I, B2, cultured *nu/nu* bone marrow cells have both NK_T and NK_M activity, whereas in conventional and *be/be* mice (Table I, B1, B3), NK_M cells appear to be entirely responsible for the cytotoxicity of bone marrow cultures against HeLa-Ms target cells.

Selective Induction of NK_I and NK_T but not NK_M or TK Subsets of Cytotoxic Effector Cells by IFN. We examined the effect of mouse IFN on the generation of three phenotypically distinct subsets described above. Normal B/6 +/+ spleen, bone marrow, or thymus lymphocytes were cultured in the presence or absence of 1,000 U/ml of partially purified IFN. After varying intervals, the cells were harvested, and their cytotoxic

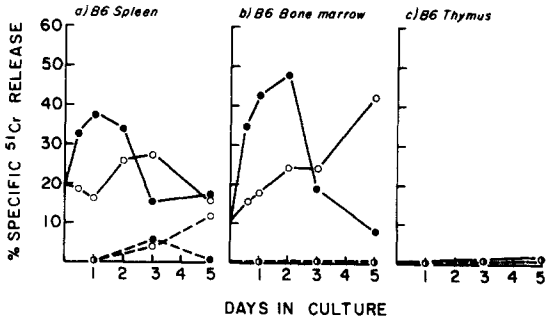

FIG. 2. Effect of IFN on the generation of cytotoxic activity in spleen, bone marrow, and thymus cell cultures of B6 mice. The cells were cultured with (●) or without (○) 1,000 U/ml of partially purified mouse IFN. At various intervals, the cells were harvested, washed, and the cytotoxicity was assayed on HeLa-Ms cells (solid lines) or P815 cells (dotted lines) at a lymphocyte to target cell ratio of 40:1. Each point represents the mean of triplicate cultures.

activity on HeLa-Ms and P815 cells was assayed. As shown in Fig. 2, IFN rapidly enhanced the cytotoxic activity of spleen and bone marrow cells on HeLa-Ms cells within 12 h after addition, reaching a peak at 1–2 d of culture. After this peak, the cytotoxic activity of the cultures rapidly declined, generally showing less activity than comparable untreated control cultures at 3–5 d. This apparent depressive effect on IFN after transient stimulation was more pronounced in bone marrow than in spleen cell cultures. The cytotoxic activity on P815 cells was not augmented by IFN and was depressed in the later phases of culture, indicating that the TK cells were not responsive to IFN.

To ascertain the cell types on which IFN was active, reexamination of the phenotype of effector cells after IFN treatment in cultures was necessary. The results presented in Table II, A1 indicate that the cytotoxicity of B/6 +/+ spleen cells was enhanced by IFN, and the phenotype of the effector cells generated remained that of the NK_I and NK_T cells, the only difference being that there was a significant increase in the percentage of these cells that were Thy-1.2$^+$ after IFN treatment. On the other hand, although the cytotoxicity of the bone marrow cell cultures was enhanced by IFN, the predominant phenotype of the effectors derived from IFN-treated cultures was found to be that of NK_T and NK_I cells (Table II, B1), whereas the phenotype of effectors obtained from cultures not treated with IFN was clearly that of NK_M cells. This result strongly suggests that the increase in cytotoxicity of bone marrow cells provided by IFN is actually a result of the differentiation of NK_I precursors into the functional effector cells, rather than of stimulation of NK_M cells.

The possibility that IFN acted on NK_M cells and converted their phenotype into that of NK_I cells was excluded by experiments shown in Table II, B3, 4. B/6 *be/be* mice lack functional effector cells of the NK_I phenotype, but have competent effector cells of the NK_M type. IFN treatment of B/6 *be/be* mouse spleens induced no significant cytotoxic activity on HeLa-Ms cells (Table II, A3), indicating that *be/be* mouse spleens functionally lacked IFN-sensitive precursor cells as well as NK_I and NK_T effector cells. Bone marrow cells of *be/be* mice (Table II, B3) showed normal cytotoxic activity on HeLa-Ms cells mediated by the NK_M phenotype, and IFN failed to enhance, and somewhat depressed, this activity. Secondly, the IFN failed to augment NK activity on the spleen and bone marrow cells of B/6 mice pretreated

TABLE II

IFN Selectively Induces the Effector Cells of the NK_I and NK_T Phenotypes

Effector source	IFN	Cytotoxicity	Percent reduction of cytotoxicity			
			Anti-Thy-1.2	Anti-Qa-5	Anti-Lyt-2.2	Anti-Ly-5.1 (plus C)
		%				
A. Spleen						
1. B/6 +/+	−	20.9	25	100	0	85
	+	46.3	55	100	0	100
2. B/6 *nu/nu*	−	14.3	30	84	0	—*
	+	60.0	72	95	0	—
3. B/6 *be/be*	−	3.5	—	—	—	—
	+	3.4	—	—	—	—
4. B/6 +/+	−	3.5	—	—	—	—
(aQa-5 plus C)‡	+	3.5	—	—	—	—
B. Bone marrow						
1. B/6 +/+	−	25.2	0	20	0	100
	+	48.8	65	70	0	100
2. B/6 *nu/nu*	−	12.6	19	31	—	—
	+	27.9	58	72	—	—
3. B/6 *be/be*	−	24.8	0	0	—	100
	+	14.9	0	0	—	100
4. B/6 +/+	−	24.8	0	0	—	—
(aQa-5 plus C)‡	+	20.4	0	14	—	—

Spleen or bone marrow cells of various mice were cultured in the presence or absence of 1,000 U/ml of IFN for 1 d. The cells were harvested, washed, and treated with C alone or various antisera plus C as described in Materials and Methods. They cytotoxicity of these cells on HeLa-Ms was assayed using a 6-h ^{51}Cr release assay at a lymphocyte to target ratio of 50:1. Assays were performed in triplicate, and each value represents the mean of two independent experiments.

* Not tested.

‡ The cells were pretreated with anti-Qa-5 plus C before the initiation of the culture.

with anti-Qa-5 plus complement (C), which eliminates both the NK_I precursor and effector cell, although NK_M activity of comparably treated bone marrow remained unaffected (Table II, B4).

We believe these results indicate that NK_I and NK_M cytotoxic cells represent distinct and independent populations and that only the former are responsive to IFN.

T Cell Growth Factor Preparation Lacking IFN Activity Can Stimulate NK_T as well as TK but not NK_I or NK_M Cytotoxic Activity. It is well established that an antigen-nonspecific growth factor (IL-2) is involved in the generation of specific alloreactive cytotoxic T cells, and can be used in establishing continuous T cell lines (14). To assess the responsiveness of the various NK cell subsets to IL-2 activity, it was crucial to separate chromatographically the IL-2 activity from IFN activity that could also augment NK activity in the cultures perhaps for different reasons. The addition of partially purified IL-2 at a final 10% concentration facilitated the generation of cytotoxicity by B/6 spleen cell cultures to both HeLa-Ms and P815 cells, although the kinetics of induction was slower than those of IFN (Fig. 3).

The serological phenotypes of the effector cells generated by the IL-2 were char-

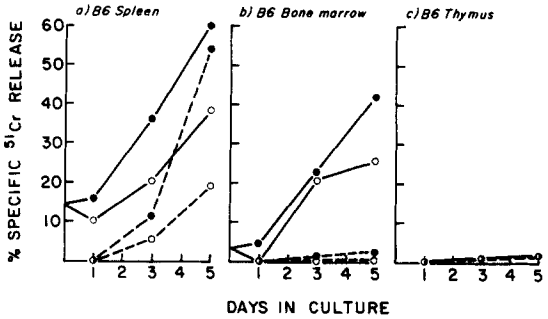

FIG. 3. Effect of partially purified IL-2 on the generation of cytotoxic activity in spleen, bone marrow, and thymus cell cultures of B6 mice. The cells were cultured with (●) or without (○) 10% vol:vol T cell growth factor. At various intervals, the cells were harvested, washed, and the cytotoxicity was assayed on HeLa-Ms cells (solid lines) or P815 cells (dotted lines) at a lymphocyte to target cell ratio of 40:1. Each point represents the mean of triplicate cultures.

TABLE III

IFN-free IL-2 Stimulates Both NK_T and TK Cells without Affecting NK_M Cell Activity

Effector source	T-F	Target cells	Cytotoxicity	Percent reduction of cytotoxicity	
				Anti-Thy-1.2	Anti-Qa-5 plus C
			%		
B/6 +/+ spleen	−	HeLa-Ms	6.3	—*	—
	+	HeLa-Ms	42.8	74	53
	−	P815	2.5	—	—
	+	P815	35.6	98	0
B/6 +/+ bone marrow	−	HeLa-Ms	19.0	0	0
	+	HeLa-Ms	38.1	67	60
B/6 aQa-5 plus C-treated bone marrow	−	HeLa-Ms	29.9	0	0
	+	HeLa-Ms	25.5	0	0

Normal B/6 spleen, bone marrow cells, and bone marrow cells pretreated with anti-Qa-5 plus C were cultured for 5 d in the presence or absence of IL-2 (10% vol/vol) in RPMI 1640-10% FCS medium. The cells were harvested, washed, and treated with C alone, anti-Thy-1.2 plus C, or anti-Qa-5 plus C. The cytotoxicity was then assayed on HeLa-Ms or P815 cells using a 6-h ^{51}Cr release assay at a lymphocyte to target ratio of 20:1. Each value represents the mean of triplicate assays.

* Not tested.

acterized, and the results are summarized in Table III. Essentially all cytotoxic activity of HeLa-Ms cells and P815 cells generated in spleen cell cultures by IL-2 was eliminated by anti-Thy-1.2 plus C. In addition, the cytotoxicity on P815 targets was totally resistant to anti-Qa-5 plus C, whereas that on HeLa-Ms cells was significantly reduced by anti-Qa-5 plus C treatment. These results indicate that IL-2 is capable of stimulating two different sets of effector cells, namely the NK_T subset of cytotoxic cells, which express Thy-1.2$^+$ and Qa-5$^+$, and TK cells, which are Thy-1.2$^+$, Qa-5$^-$. The cytotoxic activity on HeLa-Ms cells from bone marrow cultures generated by IL-2 was sensitive to both anti-Thy-1.2 and anti-Qa-5 plus C treatments (NK_T cells), whereas that in control cultures was resistant to both treatments. Because the cytotoxic activity in bone marrow pretreated with anti-Qa-5 plus C could no longer be

stimulated by the addition of IL-2 to the cultures, we infer that the NK_M cell was not responsive to IL-2. In summary, these results indicate that IFN-free IL-2 can stimulate NK_T as well as TK cell activity without affecting NK_M cell activity. Because the NK effector cells induced by IL-2 were predominantly Thy-1^+ NK_T cells, and because the alloreactive cytotoxic cells cultured from nude mouse spleens in the presence of IL-2 were totally Thy-1^+ (15), it would appear that IL-2 acts primarily, if not exclusively, on Thy-1^+ cells.

IFN and IL-2 Act on Phenotypically Different Sets of Cells. The results described above show that both IFN and IFN-free IL-2 can induce phenotypically indistinguishable cytotoxic effector cells expressing the Thy-1^+ phenotype capable of killing HeLa-Ms cells, yet the time-course of development of these effector cells suggested the mechanism of action of these two factors may be quite different. The phenotype of the cells upon which each of these factors acted remained to be established.

Normal spleen cells were pretreated with anti-Thy-1.2 plus C, with anti-Qa-5 plus C or with C alone. The remaining cells were then cultured in the presence of IFN, IL-2, or medium alone. As shown in Fig. 4, IFN enhanced the cytotoxic activity of Thy-1^+-dependent spleen cells as efficiently as in the untreated control cultures, whereas Qa-5^+-depleted cells failed to show any augmented cytotoxicity induced by IFN. Thus the phenotype of the IFN-responsive cell could be defined as the Thy-1^-, Qa-5^+, NK_I precursor cell confirming our previous results (12). In contrast, IL-2 failed to enhance the cytotoxic activity of either Thy-1.2 or Qa-5-depleted spleen cell cultures, indicating that the phenotype of the target cells of IL-2 is the Thy-1^+, Qa-5^+, NK_T cell. It thus appears that IFN can act on Thy-1.2^- population, whereas IL-2 can act only on Thy-1^+ cells. This was extended by demonstrating that IL-2

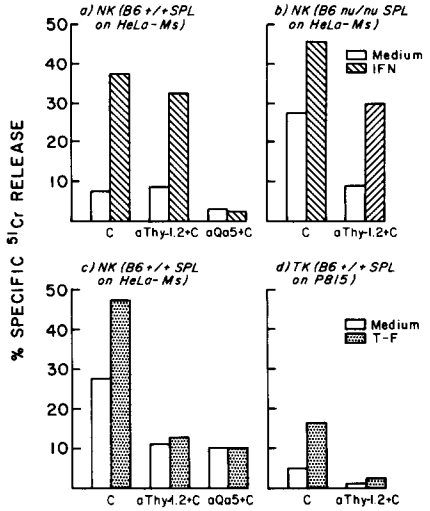

FIG. 4. Comparison of the effects of IFN and IL-2 on the generation of cytotoxic activity in the spleen cells depleted of Thy-1^+ cells or Qa-5^+ cells. Normal B6 +/+ or *nu/nu* spleen cells were treated with C alone, anti-Thy-1.2 plus C, or anti-Qa-5 plus C, washed, and then cultured in the absence (□), or presence of 1,000 U/ml IFN (▨) or 10% vol/vol T cell growth factor (▩) for 1 or 4 d, respectively. The cells were harvested, washed, and the cytotoxicity was assayed on HeLa-Ms cells (*a*, *b*, *c*) or P815 cells (*d*), using the ^{51}Cr release assay at 6 h. The lymphocyte to target cell ratio was 40:1. The means of triplicate cultures of at least three independent experiments are shown.

failed to induce TK cells from anti-Thy-1.2 plus C-pretreated spleen cells, thus confirming that IL-2 acts only on Thy-1^+ cells.

Discussion

Although it is generally accepted that NK cells constitute a functionally distinct cytotoxic defense mechanism, in most discussions of the provenance and characteristics of these cells it has been assumed that there is in fact only a single species of NK cell. A wide variety of experimental data increasingly support the view that there are multiple cell types, perhaps derived from different cell lineages, which can exert NK-like spontaneous cytotoxicity in vitro (2, 7–11, 16, 17). The aim of the present work was to delineate and characterize the diversity of functional NK cells in the mouse in terms of a number of genetically controlled surface markers. Our findings indicate that at least four phenotypically distinct classes of NK effector cells can be distinguished. At the risk of burdening an already confusing nomenclature, we found it convenient to refer to these subsets as NK_I, NK_T, and NK_M, and TK cells. The characteristics of these serologically distinguishable populations are summarized in Table IV. To this list one must add the natural cytotoxic (NC) cell that lyses solid tumor cells but not lymphomas, and expresses none of the surface markers studied here (18), although it is possible that it is related to the NK_M subset.

The existence of distinct NK effector cell types was strengthened by studies on the selective regulation of the activity of each subset by two different biological factors. In the results described here, exogenous IFN selectively induced NK_I and NK_T activity without augmenting NK_M or TK activity (Table II). We have previously found that the anti-Thy-1 sensitivity of NK activity of spleens of nude mice preinjected with virus persistently infected tumor cells (50–55%) was always greater than that of normal mice (25–35%). In data not shown, we observed a small but significant increase in anti-Thy-1-sensitive NK activity upon the addition of IFN to spleen cells in vitro. Although the results may not be definitive they suggest that IFN may have several effects on NK cells: (*a*) it was indicated that IFN can stimulate noncytotoxic Qa-5^+ Ly-5^- precursor cells to differentiate into Qa-5^+ Ly-5^+ NK_I effector cells (12);

TABLE IV

Characteristics of Four Subsets of Spontaneously Cytotoxic Cells in Mice

	NK_I	NK_T	TK	NK_M
Serological phenotype	Thy-1^-, Lyt-2^- Qa-5^+, Ly-5^+	Thy-1^+, Lyt-2^- Qa-5^+, Ly-5^+	Thy-1^+, Lyt-2^+ Qa-5^-, Lyt-5^+	Thy-1^-, Lyt-2^- Qa-5^-, Lyt-5^+
Target cell lysis				
HeLa-Ms (or YAC-1)	+	+	−	+
P815	−	−	+	−
Regulated by IFN	+	Not tested	−	−
Regulated by IL-2	−	+	+	−
Organ distribution				
Bone marrow	− (+) [−]	− (+) [+]	− (−) [−]	+ (−) [−]
Spleen	+ (+) [−]	+ (+) [+]	+ (−) [+]	− (−) [−]
	− (−) [−]	− (−) [−]	− (−) [−]	− (−) [−]
Activity in *be/be* mice	−	−	+	+
Activity in *nu/nu* mice	+	+	−	+

Parentheses activity augmented after treatment with IFN; Brackets indicate activity augmented after treatment with IL-2.

(*b*) IFN may convert a portion of NK_I to NK_T cells; and (*c*) IFN can increase the effectiveness of some NK cells in lysing targets (19).

Both IFN and a partially purified IL-2 augmented NK activity in these experiments, although they clearly acted on different subsets. IFN-free IL-2 preparations augmented the activities of TK and NK_T subsets, but not NK_I or NK_M cells. Interestingly, IL-2 augmented NK activity in spleen cells obtained from both conventional and *nu/nu* mice, but was without effect on spleen cells of *nu/nu* mice depleted of Thy-1.2^+ cells with monoclonal anti-Thy-1.2. The data of Hunig and Bevan (15) on the requirement for preexisting Thy-1^+ cells in nude mouse spleens for the development of H2-specific cytotoxic T lymphocytes (CTL) and their amplification in vitro, and Dennert's (20) development, using IL-2, of Thy-1^+ NK lines corresponding with our NK_T cells, together with the data presented here, strongly suggest that IL-2 acts primarily, if not exclusively, on Thy-1^+ cells.

One hypothesis consistent with these data would hold that IFN induces the differentiation of precursor cells into NK_I effector NK cells and subsequently converts a portion of them into NK_T effector cells. These Thy-1^+ NK_T cells could then be regulated and expanded by IL-2.

Further evidence of the independence of these subclasses derived from studies on *be/be* and *nu/nu* mice. As has been reported previously (21) *be/be* mice lack functional NK cells, but clearly possess target-binding cells, and in the present studies lacked Qa-5^+ NK cell activity, i.e., functional NK_I and NK_T cells. Beige mice possess normal TK and NK_M activity. Conventional mice depleted of Qa-5^+ cells with anti-Qa-5 plus C proved to be functionally equivalent to *be/be* mice. Conversely, *nu/nu* mice lack TK cells, but have normal NK_I and NK_T, as well as NK_M activity. Whereas spontaneous cytotoxicity of all three classes of cells was eliminated by treatment with anti-Ly-5 plus C, in the absence of complement, anti-Ly-5 selectively blocks the activity of NK_I cells, but not of NK_M or TK cells.

Two important questions clearly require further study: the relationship of these separate subsets to the conventional lymphoid cell lineages, and the role, if any, of these subclasses in immune responses in vivo. Of particular interest is the relationship between NK_I, NK_T, and TK and CTL. It remains to be established whether the NK_T cells, which can be amplified by IL-2, can differentiate further to become TK cells, which would require acquiring the Lyt-2 surface marker and losing the Qa-5 marker. The TK cells express the same phenotype as conventional CTL. It is unclear whether their activity represents polyclonal activation of conventional CTL in culture, or whether they are a T cell subset that functions physiologically as NK cells. The relationship between NK and T cells is not a trivial problem, because at issue are questions regarding differentiation of functional cytotoxic T cells through thymic and extrathymic pathways and possible antigen-specific recognition and repertoire. Finally, if one accepts the existence of at least four subclasses of cells capable of mediating spontaneous cytotoxicity in vitro, it will be important to establish the relative importance, if any, of each in vivo.

There is strong evidence that cells of the NK_I and NK_T subclasses do operate in vivo because systems have been described in which IFN induces resistance to tumor growth or virus infection in vivo (22, 23) under conditions where it is ineffective directly in vitro, suggesting that resistance in vivo must be mediated via some host cell mechanism. We have recently reported that anti-IFN serum suppresses the

resistance of *nu/nu* mice to virus persistently infected tumor cells, and that these tumors not only grow invasively in most treated mice, but also metastasize in a significant portion as well (24). Similar results using anti-asialo GM_1 antibodies reactive with NK cells have recently been observed by Habu et al. (25).

Summary

The heterogeneity of cells capable of exerting spontaneous cytotoxicity in vitro was explored using antisera to several genetically determined surface markers on mouse lymphocytes. Four phenotypes of cells derived either from fresh or cultured murine lymphoid tissue were found to exert natural killer (NK) activity in vitro. One effector cell subset, termed NK_I cells, had the serological phenotype of Thy-1^-, Lyt-2^-, Qa-5^+, and lysed measles virus persistently infected target cells (HeLa-Ms) but not P815 mastocytoma cells. It corresponds with the NK cells described in most systems in which lymphoma targets are commonly used. A second subset, with the same target cell specificity, termed NK_T is a thymus-independent cell with the phenotype Thy-1^+, Lyt-2^-, Qa-5^+, Ly-5^+. A third subset of NK cells, termed T killer (TK) cells, deriving from cultures of conventional but not nude mouse spleens, mediated spontaneous cytotoxicity of P815 mastocytoma cells, but not of virus-infected targets. It has a phenotype of Thy-1^+, Lyt-2^+, Qa-5^-, Ly-5^+, apparently identical with that of conventional, antigen-specific cytotoxic T lymphocytes. The fourth phenotype of NK cells, termed NK_M, derived primarily from cultures of bone marrow, is cytotoxic for HeLa-measles but not P815, and expresses only Ly-5^+ among the various markers tested.

Beige mice possess normal TK and NK_M activities, but were deficient in NK_I and NK_T activity. Conversely, nude mice lacked TK cells, but had normal NK_I, NK_T as well as NK_M activity. All NK cell subsets express the Ly-5 surface marker.

The existence of four phenotypically distinct NK effector cells was strengthened by studies on selective regulation of their activity by two different biological factors. Interferon (IFN) augmented NK activity of primarily one of the subsets examined, the NK_I cell; the activity of IFN on NK_T cells could not be directly tested, but IFN was without positive effect on TK or NK_M cells. In contrast, partially purified IFN-free interleuken 2 (IL-2) augmented the activities of both the TK and NK_T subsets, but not of NK_I or NK_M cell. IL-2 was active in augmenting NK activity in spleen cells obtained from both conventional and *nu/nu* mice, but was without effect on spleens of *nu/nu* mice depleted of Thy-1^+ cells. These and other data suggest that IL-2 acts primarily, if not exclusively, on Thy-1^+ cells.

These results strengthen the view that natural cytotoxicity in vitro can be mediated by several distinct cell populations under different genetic and regulatory control and indicate the importance of defining and delineating the cell lineages of each and the role of the independent subsets in resistance to virus infections and tumors in vivo.

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