CHÉDIAK-HIGASHI GENE IN HUMANS

I. Impairment of Natural-Killer Function*

By TINA HALIOTIS,‡ JOHN RODER,‡ MARGARETA KLEIN,‡ JOHN ORTALDO,§
ANTHONY S. FAUCI,‖ AND RONALD B. HERBERMAN§

*From the Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada
K7L 3N6; and the Laboratory of Immunodiagnosis, National Cancer Institute; and the Laboratory of
Clinical Investigation, National Institutes of Allergy and Infectious Diseases, National Institutes of Health,
Bethesda, Maryland 20205*

We have previously observed that a point mutation on chromosome 13 in the mouse, called beige, leads to a marked, selective impairment in natural-killer (NK)[1]-cell function (1). Because beige mice have long been used as an animal model of the Chédiak-Steinbrinck-Higashi syndrome (C-HS) in man (2) it seemed relevant to assess NK function in humans who bear this rare, autosomal recessive gene. Because of the marked propensity of C-HS patients for developing lymphoproliferative disorders that may be malignant (3) it was predicted that if NK cells in the human were important in immune surveillance, then C-H patients might have low levels of NK activity.

In this present paper we show that NK activity in two C-HS donors was profoundly impaired and did not appear to be a result of an alteration of an anti-target selectivity pattern or of the kinetics of lysis, of suppressor cells, of a lack of ability to respond to interferon, or of a lack of target-cell recognition. In the accompanying paper (4) we show that antibody-dependent cell-mediated cytotoxicity against tumor-cell targets is also depressed in C-HS patients with cytolytic activity by monocytes, by polymorphonuclear leukocytes, and by T cells that are relatively normal.

Materials and Methods

Subjects. Two male patients, Le. R. (age 26 yr), and La. R. (age: 28 yr), siblings from a consanguinous marriage, and eight different age- and sex-matched normal controls were studied in parallel experiments on three separate occasions over a 1-mo period. The detailed clinical history and many previous experiments on these particular patients has been described (5-7). At the time of study the patients were free of overt infections and were not on therapy.

[1] *Abbreviations used in this paper:* C-HS, Chédiak-Steinbrinck-Higashi syndrome; FcR⁺, with receptors for the Fc portion of IgG; FcR⁻, without receptors for the Fc portion of IgG; FCS, fetal calf serum; IF, interferon; LU, lytic unit(s); NK, natural killer; PMN, polymorphonuclear leukocytes; TBC, target-binding cells; TNP, trinitrophenyl(ated).

Cell Lines. All cell lines were maintained by continuous in vitro culture and were free of mycoplasma at the time of testing. The following cell lines were used: K562: derived from human myeloid leukemia; Molt-4: a T cell line derived from human acute lymphocytic leukemia; Alab, CEM, and MDA human breast cancer cells; TU5: SV40 virus-transformed mouse kidney cells; P815: methylcholanthrene-induced mastocytoma of mice; and human fetal fibroblasts: nontransformed primary cell culture, passaged five times.

Effector Cells. Heparinized venous blood was centrifuged (1,000 *g* for 10 min) on Ficoll-Hypaque (LSM; Litton Bionetics, Kensington, Md) and the mononuclear cell band was collected.

Cell Fractionations. Mononuclear cells were depleted of monocytes by adherence (45 min at 37°C) to plastic surfaces coated by microexudates of detached TU5 kidney cells grown to confluence as described previously (8). Cell loss was ~20% in all donors and contamination of recovered lymphocytes was <2% monocytes as judged by phagocytosis of latex beads or nonspecific esterase staining and <2% polymorphonuclear leukocytes (PMN) as indicated by Wright's-stained cytocentrifuge preparations or staining with monoclonal anti-human PMN antibody generously provided by Dr. P. Beverley, University College Hospital, London.

Mononuclear cells (30 × 10^6/ml) were also incubated for 60 min at 37°C on a 7-ml column of preswollen Sephadex G-10 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) or on a 10-ml column of scrubbed nylon wool (Fenwal Inc., Walter Kidde & Co. Inc., Ashland, Mass.) equilibrated with RPM1-1640, containing 20% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N. Y.), and nonadherent cells were washed through with three bed volumes of warm, 37°C medium. Cell recovery was 60% of input and macrophage contamination, as indicated by uptake of latex beads, was <2%.

To obtain lymphocytes with receptors for the Fc portion of IgG (FcR$^+$), mononuclear cells were depleted of monocytes by a 1-h incubation on plastic surfaces. Lymphocytes were then incubated for 60 min at room temperature in plastic flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard Calif.) precoated with trinitrophenylated (TNP) FCS and rabbit anti-TNP antibody (9). Nonadherent lymphocytes without receptors for the Fc portion of IgG (FcR$^-$) were recovered, and adherent FcR$^+$ cells were eluted with EDTA and by scraping with a rubber policeman. Both FcR$^+$ and FcR$^-$ fractions were incubated overnight at 37°C, before assay, to allow recovery of cell surface receptors.

Interferon Treatment. Lymphocytes were incubated 1 h with 10^3 units (National Institutes of Health reference standard) of partially purified human fibroblast interferon (HEM Laboratories, Rockville, Md.) or medium as control and washed two times before use.

Target-binding Assay. Lymphocyte populations, depleted of adherent cells by passage over Sephadex G-10 or nylon-wool columns, were mixed with a fivefold excess of tumor cells, centrifuged at 150 *g* for 5 min, incubated 5 min at 37°C, and then stored on ice. Pellets were gently resuspended, and the number of lymphocytes binding to tumor cells (target-binding cells [TBC]) was determined in a hemocytometer.

Cytolytic Assays. Target cells labeled with ^{51}Cr (sodium chromate) were placed together with varying numbers of effector cells in 0.2-ml microwells (Linbro Chemical Co., Hamden, Conn.) in triplicate samples. The microplates were spun at room temperature for 5 min, at 1,000 rpm, and then were incubated for 4 h. at 37°C. Supernate was measured in a gamma counter (Beckman Instruments, Inc., Fullerton, Calif.). Autolysis was determined by culturing ^{51}Cr-labeled targets alone, and total label was determined by counting an aliquot of targets after resuspension in the microwells. The following formula was used to compute the percent lysis:

$$\text{percent lysis} = \frac{\text{test counts per minute} - \text{autolysis}}{\text{total counts per minute}} \times 100.$$

Lytic Units. Lytic units (LU) were calculated from effector titration curves and 1 LU was defined as the number of effector cells required to achieve a given percent lysis.

Results

Functional Defect of NK Cells in the C-HS. In this study, two male C-HS donors and eight age- and sex-matched normal controls were studied in parallel experiments on three separate occasions over a 1-mo period. As shown in Fig. 1, C-HS peripheral
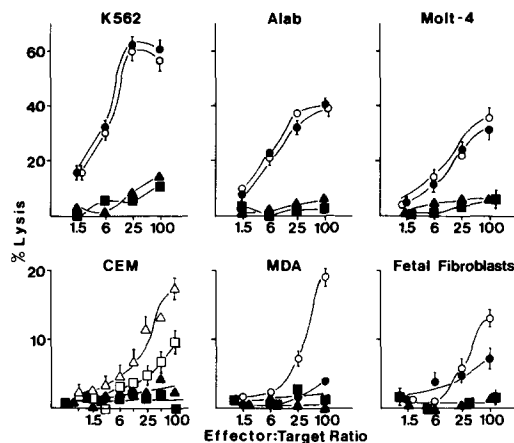
Fig. 1. NK activity in CH-S patients. Peripheral blood mononuclear cells, depleted of monocytes on microexudates were tested for cytolysis against K562, Alab, Molt-4, CEM, MDA, and human fetal fibroblast targets at varying effector:target ratios, in a 4-h ^{51}Cr-release assay. Autolysis by target culture alone was always <20%. subjects: (●) J. J. (normal); (○) J. G. (normal); ▲ Le. R. (C-HS); (■) La. R. (C-HS); (△) J. R. (normal); (□) J. O. (normal). Values represent mean percent lysis of triplicate wells ± SE. The absence of vertical bars indicates the standard error is contained within the symbol. The experiment was repeated for a total of three times for K562, three times for Molt-4, and one time for Alab, CEM, MDA, and human fetal fibroblasts. Murine lines, AT6, P815, and RBL-5 were not lysed by normal cells or C-HS donors.

blood mononuclear cells had very low cytolytic activity against K562 target cells, in contrast to the consistently high levels of the normal donors. A similarly large difference in reactivity was seen with five other target cells. The LU/10^6 calculated at 20% lysis were <1 to <0.1 for both C-HS patients tested against all targets, whereas normal donors yielded mean values of 90, 40, and 13 against K562, Alab, and Molt-4, respectively. The target cells were chosen to represent a wide spectrum, from the highly NK-sensitive, human tumor-cell lines (K562, Molt-4, and Alab) to those that are relatively resistant to lysis by some donors (MDA and CEM) or normal, nontransformed fetal fibroblasts (10). Several murine lines, (AT6, P815, and RBL-5) were not lysed by normal cells or C-HS donors. The cells from the C-HS patients had little or no reactivity against any of the targets indicating a general impairment in NK cytolytic activity. However, it should be stated that although the NK response in C-HS against these targets was significantly lower than that of controls, a small titratable response (5–10% lysis) was obtained against the most sensitive targets.

Cold-Target Competition. To investigate the specificity of the low level of cytolytic activity of the C-HS patients, a cold-target inhibition assay was performed using ^{51}Cr-labeled K562 (a highly sensitive NK target) as the hot target and unlabeled P815 (an NK-insensitive target) or unlabeled K562 as the cold targets, competing with the labeled K562. As shown in Fig. 2, natural cytotoxic activity of normal effectors was significantly ($P < 0.05$) inhibited in the presence of cold K562 targets, whereas the presence of cold P815 targets had little or no effect ($P > 0.05$). Similar trends were obtained with C-HS effectors although levels of lysis were much lower (Fig. 2, low panel). This suggests that the small residual cytolysis by C-HS effectors has the same or similar specificity as NK cells in the normal donors.

Kinetics of NK Function. One possible explanation for the low reactivity of cells from the C-HS patients was that they needed more time than allowed by the usual natural
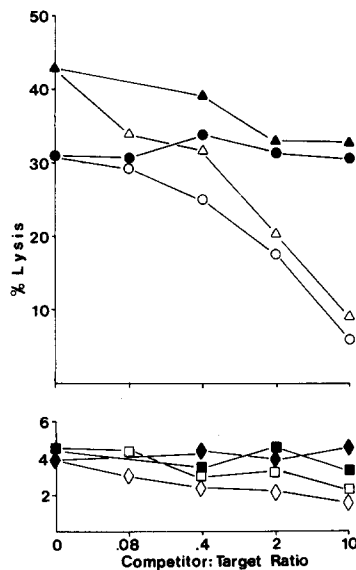
Fig. 2.   Cold-target inhibition assay. Unfractionated peripheral blood mononuclear cells were tested for cold-target inhibition in a 4-h ^{51}Cr-release assay against labeled K562 targets at varying competitor:labeled-target ratios. K562 autolysis was 6.2%. Subjects and preparations: (●) G. B. (normal) + labeled K562 + unlabeled P815; (○) G. B. + labeled K562 + unlabeled K562; (▲) B. S. (normal) + labeled K562 + unlabeled P815; (△) B. S. + labeled K562 + unlabeled K562; (■) Le. R. (C-HS) + labeled K562 + unlabeled P815; (□) Le. R. (C-HS) + labeled K562 + unlabeled K562; (◆) La. R. (C-HS) + labeled K562 + unlabeled P815; (◇) La. R. + labeled K562 + unlabeled K562.

cytotoxicity assay to express normal levels. Therefore, a kinetic study was performed in which lysis was evaluated at eight time points over a period of 17 h. As shown in Fig. 3 the NK response of C-HS donors against K562 remained quite low over this entire period. Similar results were obtained in a repeat experiment carried out for up to 24 h. of incubation. Thus, the C-HS deficiency did not appear to constitute a delay in the expression of a normal level of NK activity.

Enrichment of NK Cells.   It was important to enrich for NK cells from C-HS patients, to reduce the possibility that the apparent defect was a result of inhibition or suppression by contaminating cell types. Peripheral blood mononuclear cells were passed through a nylon-wool column or a Sephadex G-10 column. As shown in Fig. 4, column-passed cells from normal subjects exhibited an increase in lytic ability, whereas this did not occur with the cells of the C-HS patients. Fractionation of NK cells from normal donors on immune complex monolayers resulted in high NK activity in the FcR$^+$ fraction eluted from the monolayer, whereas the FcR$^-$, nonad- herent fraction exhibited little cytolytic activity. FcR$^+$ cells from C-HS donors, although slightly enriched for NK activity, yielded only low levels of NK activity comparable to the FcR$^-$ fraction from normal donors.

Absence of Suppressor Cell Activity in C-HS Cell Populations.   To further examine the possibility that the low NK reactivity of the C-HS patients might be a result of the presence of a suppressor cell rather than of a characteristic of the NK cell per se, cells from a C-HS donor were mixed with cells from a normal subject, at varying inhibitor: attacker ratios (Fig. 5). If a C-HS suppressor cell were present in the C-HS/normal
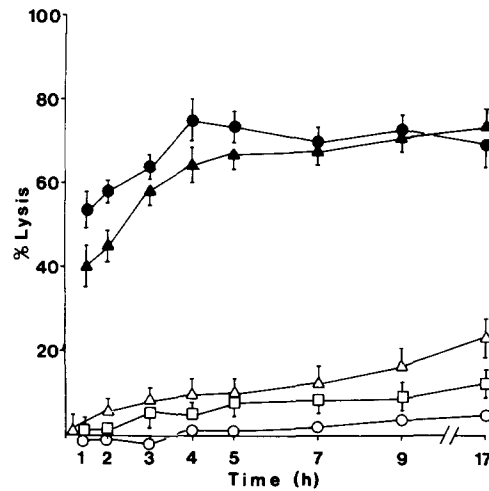
Fig. 3. Kinetics of NK activity. Peripheral blood mononuclear cells, depleted of monocytes on microexudate-coated plastic, were tested for cytolysis against K562 targets at a 25:1 effector:target ratio at varying times over a 17-h period. Values represent mean percent lysis of triplicate wells ± SD. Subjects: (●) J. J. (normal); (▲) J. G. (normal); (□) Le. R. (C-HS); (△) La. R. (C-HS); (○) autolysis.
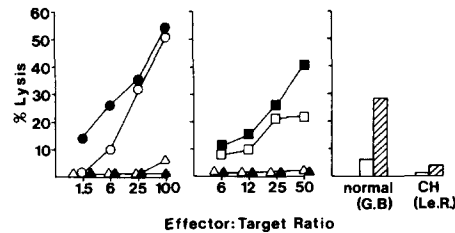

Fig. 4. NK activity in fractionated blood. Peripheral blood mononuclear cells were untreated (open symbols) or were fractionated by passage through Sephadex G-10 (left panel) or nylon-wool (middle panel) columns (closed symbols), or over immune complex-coated plates (right panel), and tested for cytolytic activity against K562 in a 4.5-h ^{51}Cr-release assay, at varying effector:target ratios. (E:T = 50:1, right panel). Values represent mean percent lysis of triplicate wells. Autolysis of target cells alone was 21.6, 7.8, and 7.9%, respectively (left to right). Subjects: circles and squares: J. G. (normal), triangles: Le. R. (C-HS). (□) FcR$^-$; (▨) FcR$^+$.

population, it might suppress the activity not only of C-HS NK cells, but of NK cells derived from the normal subject as well.

As shown in Fig. 5, C-HS cells did not inhibit cytolysis by normal cells. To rule out the possibility that suppression was masked by an enhancing allogeneic effect, cells were mixed from a normal donor and as shown, no enhancement or suppression was evident. Thus, it is unlikely that the NK defect in C-HS donors was a result of inhibitory cells.

Binding of C-HS Lymphocytes to Target Cells. In contrast to the impairment in natural killing, the frequency of nylon-passed and Sephadex G-10-passed lymphocytes binding to K562 and Molt-4 targets was essentially normal in C-HS patients (Table I). P815, an NK-insensitive target was not bound by either normal or C-HS donor cells. Therefore, the C-HS defect most likely lies within the lytic mechanism rather than within the recognition structure involved in target-effector interaction.
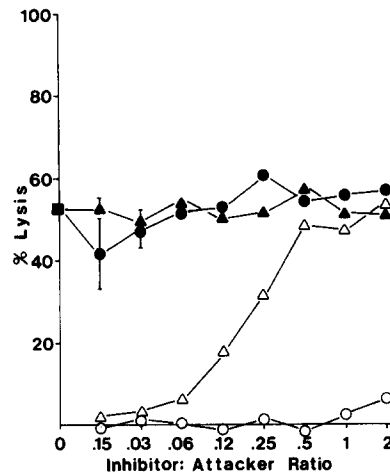
Fig. 5. Absence of suppressor cell activity in C-HS-cell populations. Varying numbers of unfractionated peripheral blood mononuclear cells from a C-HS donor, Le. R. (inhibitor), or from a normal donor, J. J., and a normal subject, J. G. (attacker), were mixed at varying ratios and then tested in a 4-h ^{51}Cr-release assay for NK activity against K562 target cells. Also shown are the results from the inhibitor cells alone added to K562. Values represent mean percent lysis of triplicate wells. Autolysis was 15.1%. Subjects: (■) J. G.; (●) J. G. + Le. R.; (○) Le. R. (control); (▲) J. G. + J. J. (control); (△) J. J. (control).

TABLE I

TBC

Experiment	Donor	Fractionation	Percent TBC		
			K562	Molt-4	P815
1	J. G. (normal)	Sephadex G-10-passed	15	37	
	Le. R. (C-HS)		15	46	—
2	G. B. (normal)	Nylon-wool-passed	20	41	1.0
	B. S. (normal)		28	34	2.6
	Le. R. (C-HS)		25	36	4.0

Ficoll-Hypaque-separated peripheral blood lymphocytes were passed over nylon-wool or Sephadex G-10 columns to remove adherent cells, and then mixed with a fivefold excess of tumor cells. Values represent the percentage of lymphocytes binding to targets.

Augmentation of NK Activity by Interferon.   Interferon (IF) has been shown to augment NK activity in humans (11) as well as in the mouse (12). As shown in Fig. 6, treatment of peripheral blood mononuclear cells for 1 h with human fibroblast IF enhanced NK activity to a variable extent in normals. Subject J. O. was boosted from 33 LU/10⁶ (calculated at 20% lysis) to 89 LU/10⁶, whereas subject J. R. was boosted to a lesser extent from 133 LU/10⁶ to 174 LU/10⁶. In the C-HS donors, IF also significantly boosted NK activity, but the levels remained much depressed compared to those of the normal donors. Activity rose from 0.02 LU/10⁶ to 1.0 LU/10⁶ for Le. R. and from 0.02 LU/10⁶ to 3.3 LU/10⁶ for La. R. Similar results were observed when Molt-4 was used as target (data not shown). These results indicate that NK cells from C-HS patients can respond to IF but with cytolytic activity which remains markedly deficient.
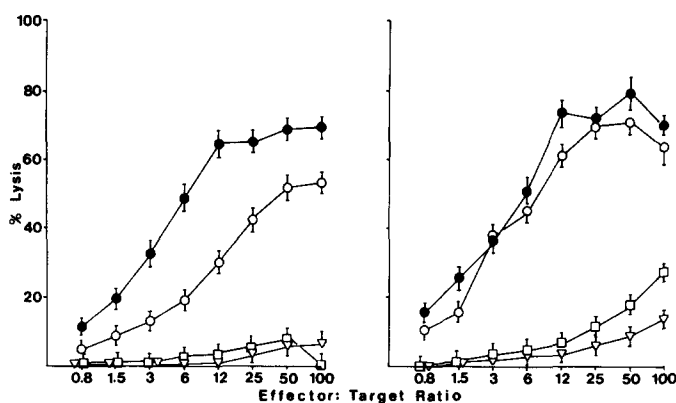
Fig. 6. The effect of IF on the NK response. The natural cytolytic activity of IF-treated cells (right panel) was compared with that of cells that had not been treated with IF (left panel) in a 4-h ^{51}Cr-release assay against K562 targets at varying effector:target cell ratios. Values represent mean percent lysis of triplicate wells ± SD Autolysis was 5.4%. Subjects: (●) J. R. (normal); (○) J. O. (normal); (∇) Le. R.; (□) La. R. (C-HS).

TABLE II

In Vitro Generation of NK Activity

Donors	Without stimulators		With stimulators
	Control	IF	
	*LU/10⁶**		
B. S. (normal)	84	131	150
G. B. (normal)	12	21	41
Le. R. (C-HS)	<0.1	<0.1	<0.1
La. R. (C-HS)	<0.1	<0.1	<0.1

Peripheral blood mononuclear cells were cultured 7 d with or without a pool of irradiated stimulator cells from four normal donors as described previously (13). Effectors were harvested, treated with or without IF, and tested in a 4-h ^{51}Cr-release assay against K562 cells.

* Calculated as the number of cells required to produce 30% specific lysis.

In Vitro Generation of NK Activity. It was of interest to determine whether the defective NK activity of C-HS patients might be reversed during in vitro culture. Peripheral blood lymphocytes were cultured 7 d as described previously (13). In the absence of stimulator cells, normal donors exhibited 12–84 LU/10⁶ against K562 target cells, whereas C-HS donors had <0.1 LU/10⁶ (Table II). IF pretreatment of cultured effector cells enhanced NK activity almost twofold in normal cells but did not induce detectable activity in the cells from the C-HS donors. Furthermore, normal cells, but not C-HS cells, cocultured with a pool of irradiated stimulators showed significant enhancement of NK activity against K562. These results suggest that the C-HS defect in NK function persists in vitro and may even be exacerbated because cultured lymphocytes from C-HS donors had no detectable response to IF.

Discussion

The overall conclusions from the data presented was that NK activity was profoundly depressed in the two cases of C-HS that were available for study.

Spontaneous cytolytic activity of nonadherent, FcR^+ lymphocytes against several NK-sensitive targets was at least 100-fold greater in age- and sex-matched normal donors compared to the C-HS donors, in short-term, 4-h ^{51}Cr-release assays. The low NK activity in C-HS donors was not simply a result of a delayed response because the defect was still evident in a 17- to 24-h cytolytic assay. In addition, we screened both adherent and suspension target-cell lines of both transformed and nontransformed phenotypes selected to represent a wide spectrum of NK-susceptible target cells. Therefore, the defective C-HS response was not simply a result of a shift in the selectivity of cytotoxic activity. It should be noted that although the NK response of C-HS donors was low, it was not entirely absent. C-HS effector cells mediated cytolysis of K562, the most sensitive NK target known, and sometimes reached levels of 15% lysis at the highest effector:target ratio (Fig. 1); this response titrated and increased with time of incubation (Fig. 3). In addition, the NK response in both normal cells and C-HS donors was inhibited in cold-target competition assays. The low C-HS response was boosted significantly by IF but the levels after treatment remained very low relative to those seen with normal donors. The possibility that this response is not a result of residual NK cells but rather may represent contaminating promonocytes, which also exhibit IF-boostable, NK-like activity, at least in the mouse, cannot be excluded (14).

We then asked whether the low NK response in C-HS donors was a result of suppressor cells. When low-responder C-HS cells were mixed with normal cells at ratios as high as 2:1, no suppression of cytolysis was observed. In addition, the removal of adherent cells, a suggested source of NK suppressors (15), did not restore the NK response in C-HS donors. Furthermore, the C-HS defect persisted after 7 d of in vitro culture in the absence of host humoral factors. Therefore it is unlikely that the C-HS defect is a result of suppressor cells or factors.

The nature of the NK defect is not entirely known, but may lie within the lytic pathway rather than at the level of the recognition structure or NK population size. Hence, the frequency of lymphocytes binding to NK-sensitive targets was normal. The majority of TBC (80%) detected in this assay have been shown to lyse their targets in the mouse (16–17). In the human, preincubation of effectors with solubilized glycoproteins from NK-sensitive targets specifically inhibits the formation of TBC by 50% (18). Although not all human TBC are cytolytic NK cells, the proportion of TBC that are NK cells is large enough to detect any differences in C-HS donors. Because TBC frequency was normal in C-HS patients, we can assume that NK frequency may also be normal.

In summary, we have described the first primary immunodeficiency disorder in humans selectively involving NK cells alone as shown in the following paper (4). Other studies of the more familiar immunodeficiencies failed to reveal significant defects in NK function (19), except in the case of severe combined immunodeficiency (20).

It is interesting to note that C-HS patients surviving early infections almost invariably succumb to lymphoproliferative disorders that some investigators feel are malignant (3). The finding of a marked deficiency of NK function provides a possible explanation for this and is consistent with the suggested role of NK cells in immune surveillance (21). If NK deficiency leads to malignancy, then IF therapy may benefit these patients because we have shown here that C-HS cells treated in vitro with IF

have enhanced NK activity. In addition, because we are unaware of reports of other nonlymphoid tumors in these C-HS patients, then these observations may restrict the validity of the NK surveillance theory to the lymphoreticular system.

Summary

Natural-killer (NK)-cell function was profoundly depressed in donors homozygous for the Chédiak-Steinbrinck-Higashi syndrome (C-HS) gene when compared with age- and sex-matched normals. This apparent defect was not simply a result of a delayed response because little cytolysis was evident in kinetics experiments even after 24 h of incubation. NK cells from C-HS donors failed to lyse adherent (MDA, CEM, and Alab) or nonadherent (K562 and Molt-4) tumor cell lines or nontransformed human fetal fibroblasts. Therefore, the apparent C-HS defect was not a result of a shift in target selectivities. In addition, the depressed reactivity did not appear to be a result of suppressor cells or factors because: (a) enriched NK populations (nonadherent lymphocytes bearing receptors for the Fc portion of IgG) from C-HS donors were low in NK-cell function, (b) C-HS mononuclear cells did not inhibit the cytotoxicity of normal cells in coculture experiments, and (c) cells from the C-HS donors remained poorly reactive even after culture for up to 7 d. The nature of the defective NK activity in C-HS patients is not clear but may lie within the lytic mechanism rather than at the level of the recognition structure or population size because the frequency of target-binding cells was normal. In vitro NK activity could be partially restored by interferon treatment. Combined with the results presented in the following paper (4), these observations suggest that the C-HS gene causes a selective immunodeficiency disorder, mainly involving NK cells. This finding, in conjunction with the high incidence of spontaneous possibly malignant, lymphoproliferative disorders in these patients, may have important implications regarding the theory of immune surveillance mediated by NK cells.

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