CHÉDIAK-HIGASHI GENE IN HUMANS

II. The Selectivity of the Defect in Natural-Killer

and Antibody-dependent Cell-mediated Cytotoxicity Function*

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In the preceding (1) paper we showed that human Chédiak-Steinbrinck-Higashi syndrome (C-HS)[1] donors had a marked impairment in natural-killer (NK)-cell function. It was then relevant to ask whether this defect was selective for NK cells as previously observed in the mouse C-HS model (2) or whether other effector-cell types were also defective. Because the C-HS is thought to involve a single recessive gene (3), it was hoped that these experiments would help clarify the relationship between NK cells and a variety of other cell types capable of lysing tumor-cell targets, including K cells involved in antibody-dependent cell-mediated cytotoxicity (ADCC), T cells, monocytes, and neutrophils. In this paper, we show that lymphocyte-mediated ADCC against tumor-cell targets is also defective, whereas ADCC mediated by both mononuclear cells and polymorphonuclear leukocytes (PMN) against erythrocyte targets is normal. Cytolysis of tumor cells by other effectors (T cells, monocytes, and PMN) was also relatively normal. These results suggest that C-HS patients may have a quite selective impairment in immunologic function. The implication of these findings for immune surveillance is discussed.

Materials and Methods

The patients and most of the procedures and materials used have been detailed in the preceding paper (1).

Additional Cell Lines. Additional cell lines used were: Daudi: human B lymphoblastoid cell

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[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; C-HS, Chédiak-Steinbrinck-Higashi syndrome; ConA, concanavalin A; E:T, effector:target; [³H]Tdr, [³H]thymidine; LU, lytic units; NK natural killer; PHA, phytohemagglutinin; PMN, polymorphonuclear leukocytes; PWM, pokeweed mitogen; RBC, erythrocyte(s).

line; Chang: a cell line derived from human hepatocarcinoma; RBL-5: murine T lymphoma induced by Rauscher leukemia virus; P815: mastocytoma induced by methylcholanthrene; TU5: SV40 virus-transformed murine kidney cells.

Lymphocyte Preparation. Peripheral blood was separated by centrifugation (1,000 *g*) over Ficoll-Hypaque (Litton Bionetics, Kensington, Md.) and the mononuclear cell band was depleted of monocytes by adherence (45 min at 37°) to fibronectin microexudates of detached TU5 kidney cells grown to confluence. Cell loss was ~20% in all donors and contamination of the recovered lymphocytes was <2% monocytes as judged by phagocytosis of latex beads or nonspecific-esterase staining and <1% PMN as indicated by Wright's-stained cytocentrifuge preparations or by staining with a specific monoclonal anti-human PMN antibody generously provided by Dr. P. Beverley, University College Hospital, London.

Polymorph Preparation. Cell pellets from Ficoll-Hypaque centrifugation were sedimented on dextran followed by recentrifugation on a Ficoll-Hypaque percoll mixture as previously described (4). Purified populations were 99–100% PMN as indicated by morphology and fluorescent staining with a monoclonal human anti-PMN antibody.

Monocyte Preparation. Ficoll-Hypaque-purified mononuclear cells were incubated on microexudates of detached TU5 cells grown to confluence, and adherent cells (4–7% of total) were recovered by EDTA treatment and vigorous shaking (5, 6). Recovered cells were >90% positive for latex uptake and for staining with nonspecific esterase.

ADCC. Lymphocytes were tested in a 4-h ^{51}Cr-release assay against NK-insensitive (RBL-5) and relatively low-NK-sensitive targets (Daudi and Chang), which had been pretreated 1 h at 20°C with a predetermined optimum concentration of antibody, and washed. RBL-5 was pretreated with a 1:10 dilution of rabbit anti-mouse thymocyte serum. Daudi, which expresses surface IgM, was pretreated with a 1:50 dilution of rabbit anti-human IgM (kappa) serum and Chang cells were pretreated with a 1:10 dilution of hyperimmune rabbit anti-Chang cell serum. In some experiments, mononuclear cells and purified PMN were tested for ADCC activity in an 18-h assay against ^{51}Cr-labeled human erythrocytes pretreated with a 1/10 dilution of rabbit IgG anti-human erythrocyte (RBC) antibody.

Lectin-induced Cytotoxicity. Purified lymphocytes or PMN were tested in an 18-h ^{51}Cr-release assay against NK-insensitive targets (P815 and RBL-5) in the presence of phytohemagglutinin (PHA) (PHA-P, The Wellcome Research Laboratories, Beckenham, Kent, England).

Growth-inhibiton Assay (Cytostasis). Target cells were plated at a concentration of 2 × 10⁴/ sample and incubated with varying numbers of PMN. After 24 h at 37°C, cultures were pulsed with 1 µCi of [³H]thymidine ([³H]Tdr) for 12 h and then harvested on a Mash harvester, (Microbiological Associates, Walkersville, Md.) and counted in a Packard liquid scintillation counter (Packard Instrument, Inc., Downers Grove, Ill.). The percentage inhibition of [³H]Tdr uptake was calculated according to the formula $(1 - A/B) \times 100$, where A was the radioactivity incorporated in the presence of effector cells, and B was the isotope uptake in control cultures without effectors. Granulocytes took up <5% of the isotope and the inhibition of isotope uptake was not a result of cold thymidine release (4).

Cytolytic Assays. Cytolysis in short-term (<24 h) assays was measured by ^{51}Cr release from labeled targets as described previously in detail (2). The percent lysis was calculated as:

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

where the autolysis of targets was measured in the absence of effectors and the total counts per minute were measured in an aliquot of labeled targets. Cytolysis in long-term (48 h) assays was measured by [³H]Tdr release from target cells prelabeled 18–24 h with [³H]methyl thymidine. The percent lysis was calculated as A/B × 100, where A was the counts per minute in the test sample and B was the sodium dodecyl sulfate-releasable radioactivity. Autolysis by target cells alone (always <33%) was subtracted from this value.

Results

Deficient ADCC in C-HS Donors against Tumor Targets. As shown in Fig. 1 (upper panels), peripheral blood lymphocytes, depleted of monocytes, from the two C-HS
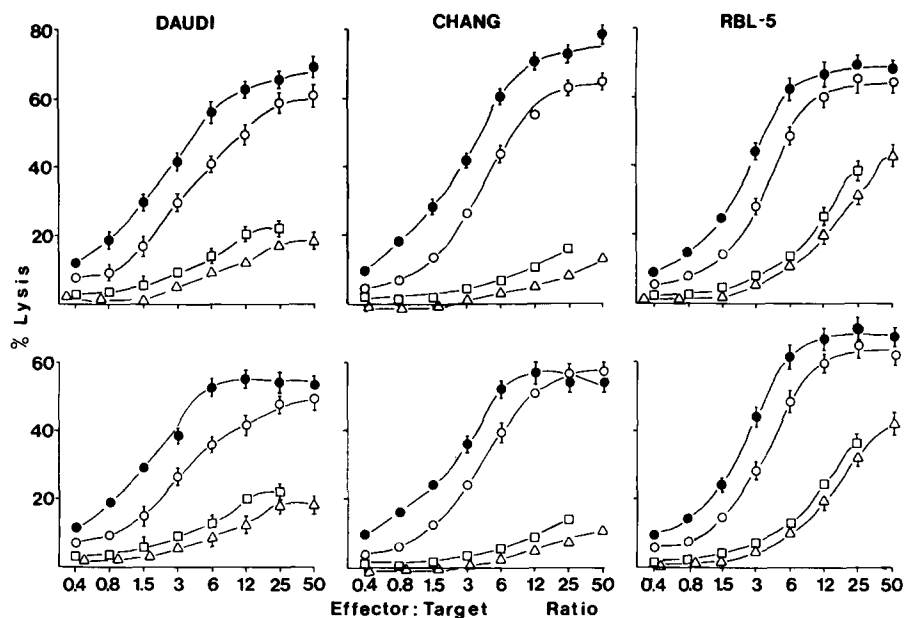
Fig. 1. ADCC against tumor cell targets. Peripheral blood mononuclear cells, depleted of monocytes on microexudates, were tested for ADCC against Daudi, Chang, and RBL-5 cells that were pretreated with rabbit antibody against human IgM (kappa), Chang, and mouse thymocytes, respectively, in a 5-h ^{51}Cr-release assay at varying effector:target (E:T) ratios. Values represent mean percent lysis of triplicate wells ± SE against antibody-treated targets before (upper panels) or after (lower panels) subtraction of the percent lysis for non-antibody-treated targets. Autolysis of antibody-treated targets alone was 7.1, 8.5, and 18.9% for Daudi, Chang, and RBL-5, respectively. Subjects: (●) J. R. (normal); (○) J. O. (normal); (□) La. R. (C-HS) (△) Le. R. (C-HS).

donors were markedly impaired in their capacity to lyse three antibody precoated tumor-cell lines. Because there was some lysis of non-antibody-coated targets as well, as a result of NK effects, the lytic values for antibody-coated and uncoated targets have been subtracted (Fig. 1, lower panel) to give values reflecting only ADCC. In the representative experiment shown, the two normal subjects had a mean value of 170 lytic units (LU)/10^6 against Daudi and 125 LU/10^6 against Chang, compared to 10 LU/10^6 and 4 LU/10^6, respectively, for the C-HS donors when calculated at the 20% lysis level. This reflects an average 24-fold deficiency in ADCC lytic capacity against human tumor cells by C-HS lymphocytes.

Lysis of antibody-coated RBL-5, a murine tumor, was also impaired in C-HS donors although for unknown reasons, the impairment was less pronounced than that seen against human tumors (Fig. 1, right panel). The difference between normal (131 LU/10^6) and C-HS lymphocytes (16 LU/10^6) was only eightfold, based on LU. These ADCC experiments were repeated on three separate occasions with two C-HS patients, and similar results were obtained with six different age- and sex-matched normal donors.

Fractionation procedures revealed that the effector cells mediating ADCC were nonadherent lymphocytes with receptors for the Fc portion of IgG (FcR$^+$) (Table I). In addition, removal of adherent cells without receptors for the Fc portion of IgG (FcR$^-$) did not restore the C-HS ADCC response beyond a normal enrichment effect.

TABLE I

Fractionation of ADCC Effector Cells

Experiment	Fractionation	Normal (J. G.)	C-HS (Le. R.)
		LU/10^6§	
1	Unpassed*	97 (59)	<1 (10)
	Sephadex G-10 passed	133 (63)	4 (15)
2	FcR⁻‡	ND ‖ (0)	ND (0)
	FcR⁺	ND (22)	ND (2)

* Peripheral blood mononuclear cells were passed through Sephadex G-10 columns and compared in a 4-h ^{51}Cr-release assay at various E:T ratios against Daudi target cells that were precoated with rabbit anti-human IgM (kappa).

‡ Peripheral blood mononuclear cells were depleted of monocytes by adherence to plastic and then incubated on immune-complex-coated plastic dishes. Nonadherent lymphocytes (FcR⁻) were recovered in the supernate and adherent (FcR⁺) cells were eluted with EDTA and scraping. Both fractions were incubated overnight and then assayed for 4 h against RBL-5 targets that were precoated with rabbit anti-thymocyte serum.

§ Calculated at 15% lysis, and the numbers in brackets represent the percent lysis at a 50:1 E:T ratio.
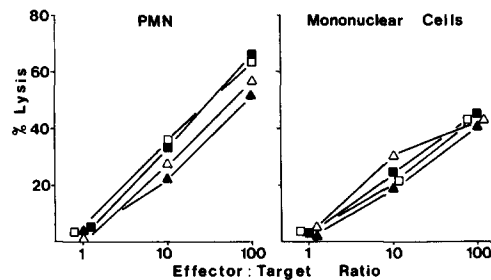
‖ ND, not done.

FIG. 2. ADCC against erythrocyte targets. Mononuclear cells (74% lymphocytes and 23% monocytes) were obtained by Ficoll-Hypaque separation, and the lymphocytes consisted of ~72% E-rosette-forming cells (T cells), 10% surface-Ig-positive cells (B cells), and 27% FcR⁺ cells (IgG) with no significant differences between donors. PMN were obtained by dextran sedimentation of Ficoll-Hypaque pellets and consisted of ~80% FcR⁺ cells, 90% of which phagocytosed bovine erythrocytes coated with rabbit anti-bovine RBC antibody (IgG). Effector cells were tested in an 18-h ^{51}Cr-release assay against human RBC (type A⁺) precoated with a 1/10 dilution of rabbit IgG anti-human RBC antibody. Subjects: (▲) normal 1; (■) normal 2; (△) La. R. (C-HS); (□) Le. R. (C-HS).

Therefore, the defect in ADCC by C-HS cells is not likely a result of suppressor cells.

Normal ADCC against RBC Targets in C-HS Donors. The results in Fig. 2 show that the failure of lymphocytes to efficiently lyse tumor cells (Fig. 1) was not a result of a generalized block in the ADCC mechanism because mononuclear effector cells and PMN from C-HS donors lysed antibody-coated human RBC erythrocytes in a normal fashion.

Spontaneous Lysis by Monocytes and Cytostasis by PMN are Normal in C-HS Donors. As shown in Table II monocytes, purified on the basis of adherence to microexudates, caused low but significant lysis of [³H]Tdr-labeled tumor cells (TU5) when tested in a 48-h assay. Levels of lysis were not different in the two normal and two C-HS

TABLE II

Spontaneous Lysis of Tumor Cells by Monocytes

Donors	Percent lysis	
	20:1 E:T ratio	10:1 E:T ratio
J. G. (normal)	15.0	12.4
J. J. (normal)	ND*	13.6
La. R. (C-HS)	ND	12.8
Le. R. (C-HS)	13.0	ND

Ficoll-Hypaque-purified mononuclear cells were incubated on microexudates, and adherent cells (4–7%) were recovered by EDTA treatment and vigorous shaking. Effectors (>90% latex positive, and nonspecific esterase positive) were tested in a 48-h [^{3}H]Tdr-release assay against TU5 target cells (SV40 virus-transformed mouse kidney cells).

* ND, not done.

TABLE III

Spontaneous Cytostasis of Tumor Cells by PMN Leukocytes

Donor	Percent cytostasis	
	20:1 E:T ratio	10:1 E:T ratio
J. O. (normal)	33	ND*
J. G. (normal)	32	9
J. J. (normal)	39	20
La. R. (C-HS)	26	19
Le. R. (C-HS)	33	18

Cell pellets from Ficoll-Hypaque were sedimented on dextran followed by recentrifugation on a Ficoll-Hypaque-percoll mixture (4). Purified populations were 99% PMN as indicated by morphology and staining with monoclonal anti-human PMN antibody. Effectors were tested in a 36-h cytostasis assay as measured by inhibition of [^{3}H]Tdr uptake during a 12-h pulse. Cytostasis against K562 was not inhibited by catalase (4).

patients tested. PMN, purified from peripheral blood by dextran sedimentation, were also tested for cytostasis of K562 target cells. As shown in Table III levels of cytostasis were not different between the C-HS donors and normals.

Lectin-induced Killer Cells. Lectin-dependent cytolysis of tumor cells by peripheral blood lymphocytes has been shown to be mediated largely by T cells (7) of both Tμ and Tγ types (8) and is thought to reflect the cytolytic potential of the total T cell pool (9). As shown in Fig. 3, PHA-dependent lysis of both P815 and RBL-5 was entirely normal in one C-HS donor (Le. R.) but was low in the other (La. R.), compared to four age- and sex-matched normal donors. The dose of PHA used was optimum and all donors had a similar threshold for stimulation (Fig. 4).

Neutrophils are also capable of lysing certain tumor cells in the presence of lectin (10). As shown in Table IV, lysis of RBL-5 by purified PMN from C-HS donors in the presence of PHA was within the normal range.

Mitogen-induced Proliferation. As shown in Fig. 5, [^{3}H]Tdr uptake in C-HS lymphocytes was normal in response to the T-dependent lectins, concanavalin A (ConA) and PHA and the B- and T-dependent lectin pokeweed mitogen (PWM) in confirmation of earlier reports (11). The magnitude of the peak response, the mitogen threshold for
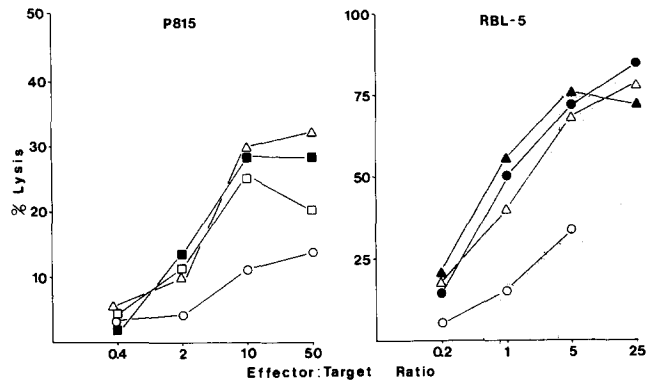
Fig. 3. Lectin-dependent cytolysis by lymphocytes. Lymphocytes were depleted of monocytes on microexudates and were tested in an 18-h ^{51}Cr-release assay against P815 (mouse mastocytoma) or RBL-5 (Rausher leukemia virus-induced murine lymphoma) targets in the presence of 2.0 μg/ml PHA. No lysis occurred in the absence of PHA, and these concentrations were not toxic. Cytolysis was not apparent at 4 h of incubation. Values represent mean percent lysis in triplicate wells. Standard error values were <10% of the mean. Subjects: (●) J. J., (▲) J. G., (■) B. S., and (□) G. B., all normal; (△) Le. R. and (○) La. R., both C-HS.
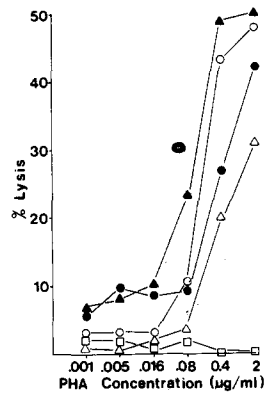

Fig. 4. Lectin titration in lectin-dependent cytolysis. Peripheral blood lymphocytes were tested in an 18-h ^{51}Cr-release assay against P815 targets at a 25:1 E:T ratio in the presence of varying concentrations of PHA. Values represent mean percent lysis of triplicate wells. Standard error values were <10% of the mean. Subjects: (●) G. B. and (▲) B. S., normal; (○) Le. R. and (△) L. R., C-HS; (□) autolysis in the presence of PHA.

a detectable response, and the optimum mitogen dose was similar in C-HS and normal donors, thereby suggesting normal B and T cell response to mitogenic stimuli.

Discussion

These results suggest that the C-HS defect in cellular cytotoxicity is selective for effector lymphocytes mediating ADCC against tumor cells.

In the preceding paper (1), we showed that NK cytolysis was impaired >400-fold in terms of LU compared to a 24-fold defect in ADCC as shown here (Fig. 1). These findings suggest that some, but not necessarily all, lymphocyte ADCC activity is mediated by NK cells. This hypothesis is supported by the work of others (12) who find striking parallels in NK and ADCC activity in the human (12). For example in

TABLE IV

Lectin-dependent cytolysis by PMN Leukocytes

Donor	Percent lysis	
	5:1 E:T	1:1 E:T
J. J. (normal)	60	33
J. G. (normal)	25	18
La. R. (C-HS)	50	26
Le. R. (C-HS)	51	22

Peripheral-blood PMN leukocytes (99% pure) were tested in an 18-h cytolytic assay against RBL-5 in the presence of 6 μg/ml PHA. No lysis occurred in the absence of PHA or in shorter assay-incubation periods such as 4 h.
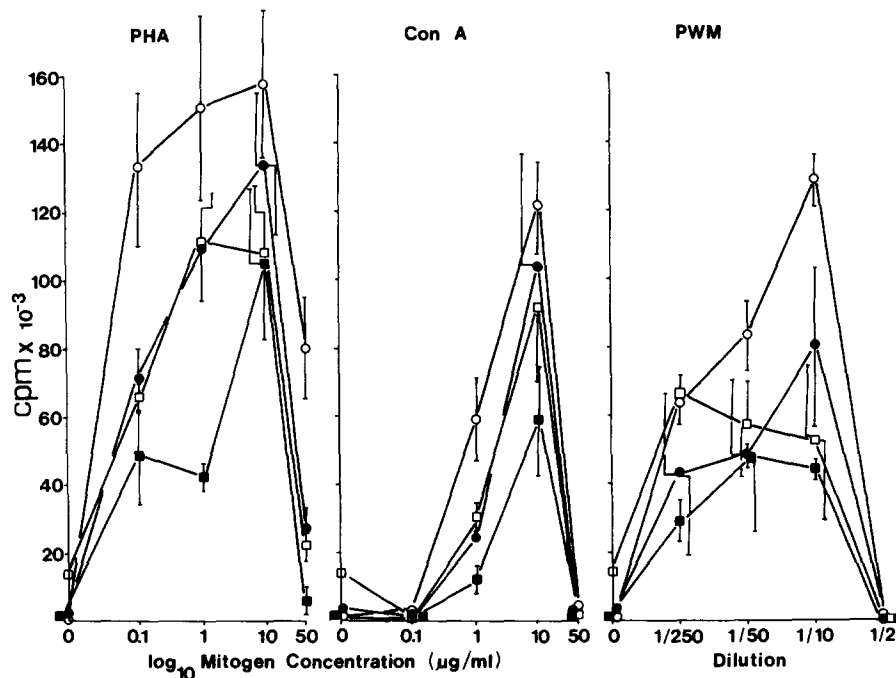

FIG. 5. Peripheral blood mononuclear cells, depleted of monocytes on microexudate plates, were assayed for mitogen-induced proliferation in the presence of varying concentrations of PHA, ConA, or PWM. Cells were in culture for a total of 4 d and were pulsed with [³H]Tdr ~30 h before harvest. Values represent mean counts per minute ± SD of triplicate wells. Control values in unstimulated wells were ~1,000 ± 100 cpm for subjects J. R., J. O., and Le. R., whereas La. R. yielded a value of 14,845 ± 655 cpm. Subjects: (O) J. R. and (●) J. O., normals; (□) La. R.; and (■) Le. R., C-HS.

our study, both NK (1) and ADCC (Table I) effectors were FcR⁺, nonadherent lymphocytes. In additional experiments, removal of FcR⁻ cells or adherent cells did not restore the ADCC defect in cells from C-HS donors, thereby suggesting that adherent suppressor cells, such as those described by Cudkowicz and Hockman (13), were not solely responsible for the C-HS defect. Furthermore, the defective ADCC of tumor-cell targets did not reflect a generalized failure in the ADCC mechanism because ADCC against antibody-coated human RBC mediated by PMN or mononuclear cells was normal (Fig. 2). As shown by others, ADCC against RBC by a

mononuclear fraction is largely mediated by monocytes (14). Further experiments also revealed that the defect in NK-lymphocyte-mediated lysis shown in the preceding paper (1) was not a result of a generalized failure of the spontaneous lytic mechanism because purified monocytes from C-HS donors lysed tumor targets in a normal fashion (Table II). In addition, PMN from C-HS donors exhibited normal growth inhibitor effects when assayed against K562, an NK-sensitive target. Cytostasis in this system was previously shown to be catalase insensitive (4). These findings, together with the observation of normal PMN-mediated, lectin-dependent cytolysis of tumor cells (Table IV) by C-HS donors are surprising because the principal defect in C-HS patients recognized to date has been a morphological and functional abnormality of granules in their granule-containing cells, most readily recognizable in the PMN (3, 15). This suggests that the enzymes that are deficient in C-HS granules such as elastase (16) may not be important in PMN-mediated cytolysis or cytostasis. It is also interesting to note that it is the primary or azurophil granules which are defective in C-HS granulocytes (15). Because ConA induces the release of cytolytic extracellular enzymes from azurophil granules (10), as well as specific granules, (17) in normal cells, our results showing normal lectin-dependent cytolysis by PMN further suggest that these enzymes (myeloperoxidase) are excreted normally in spite of the abnormal structure of azurophil granules. In addition, metabolic H_2O_2 must also be generated normally in C-HS PMN because this agent is necessary for lectin-induced cytolysis by PMN (10).

T cell-effector function in purified lymphocyte populations was indirectly assessed by lectin-dependent cytolytic assays that have been shown to solely measure cytotoxic T cells in the human (7, 8). One C-HS patient (Le. R.) responded normally to two different, NK-insensitive targets, whereas the other patient (La. R.) was deficient. Therefore a definitive statement on the status of cytolytic T cells in these patients awaits a larger study. However, T cell-mediated immunity in general was previously shown to be normal in these and other patients as judged by delayed hypersensitivity to mumps, candida, dinitrochlorobenzene, and streptokinase-streptodornase, and by proliferative responses to T-dependent mitogens, ([11, 18, 19]; Fig. 5). In addition, T cell immunity in beige mice, the animal model for the C-HS syndrome, was relatively normal with respect to rejection of skin grafts, lectin-dependent T cell killers, and alloimmune or mixed-lymphocyte-culture-generated cytotoxic lymphocytes (2).

In summary, these results show that the cytolytic defect in C-HS donors is selective for NK-ADCC cells and does not appear to involve other effector cell types. This implies that the lytic mechanisms involved in NK and ADCC are distinct from those mediated by other effector cells (T cells, monocytes, and PMN). The existence of patients with such a selective effector-cell defect affords a unique opportunity to delineate the actual steps involved in the lytic pathway utilized by NK or K cells. Furthermore the occurrence of this selective immunological defect in C-HS patients, who are known to have a high incidence of lymphoproliferative disease (20), suggests that NK-ADCC cells may play an important role in immune surveillance in man.[2]

Summary

Antibody-dependent cell-mediated cytolysis (ADCC) of human tumor cells by

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[2] Since submission of the manuscript, we have been able to completely restore the defective NK response in C-HS patients by a short preincubation of PBL with cyclic guanosine monophosphate (P. Katz. Unpublished result.).

FcR$^+$ nonadherent effector lymphocytes as well as natural killer (NK) activity was markedly impaired in Chédiak-Steinbrinck-Higashi Syndrome (C-HS) patients. Compared to a >400-fold defect in NK activity in terms of lytic units, the abnormal ADCC response in C-HS donors was 24-fold below normal suggesting a partial but not complete overlap of lymphocytes or lytic mechanisms responsible for ADCC and NK. The ADCC mechanism against erythrocyte targets, however, was normal, thereby suggesting a qualitative difference in these two forms of ADCC. Other effector-cell functions against tumor-cell targets were normal as measured by (a) spontaneous cytolysis mediated by monocytes, (b) spontaneous cytostasis mediated by neutrophils, and (c) lectin-dependent cytolysis mediated by neutrophils. Although one C-HS patient was low in lectin-dependent cytolysis mediated by lymphocytes, the other C-HS patient was normal, thereby suggesting that cytolytic T function was not linked to the NK-ADCC defect. In addition, the proliferative response to T-dependent mitogens was also relatively normal. These results, combined with other studies showing normal cell-mediated and humoral immunity in these same patients, suggest that patients with C-HS have an immunodeficiency which is selective for NK and ADCC activity.

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