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Biology of Natural Killer Cells

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I. Introduction

In 1960 Govaerts (1) observed that thoracic duct lymphocytes of dogs carrying a kidney transplant were cytotoxic *in vitro* for kidney cells of the donor animal. Since then the study of cytotoxic lymphocytes has been extended to various cellular reactions of adaptive immunity, directed against transplantation antigens on allogeneic cells, viral antigens, tumor-associated antigens, and self-antigens in autoimmune pathology (2, 3). The specific adaptive cytotoxic response against transplantation alloantigens is mediated by thymus-dependent effector T lymphocytes. The alloantigens recognized by the cytotoxic T lymphocytes (CTLs) were shown to be encoded by genes of the major histocompatibility complex (MHC) (4) and were later identified as the products of MHC class I and class II genes (5–7).

Numerous studies of humans and experimental animals have tested the hypothesis that CTLs directed against antigens expressed *de novo* on syngeneic tumor cells were responsible for the immune surveillance against growth of neoplastic cells (8). In these studies, cytotoxicity mediated by lymphocytes of cancer patients was demonstrated on both autologous and allogeneic tumor target cells. However, Zinkernagel and Doherty (9) showed that CTLs recognize viral antigens on target cells only in association with products of syngeneic MHC, and this MHC restriction of CTLs was also then demonstrated for tumor-associated antigens (10).

On the other hand, studies of cytotoxicity by human lymphocytes revealed not only that both allogeneic and syngeneic tumor cells were lysed in a non-MHC-restricted fashion, but also that lymphocytes from normal donors were often cytotoxic. Lymphocytes from any healthy donor, as well as peripheral blood and spleen lymphocytes from several experimental animals, in the absence of known or deliberate sensitization, were found to be spontaneously cytotoxic *in vitro* for some normal fresh cells, most cultured cell lines, immature hematopoietic cells, and tumor cells (11–16). This type of nonadaptive, non-MHC-restricted cell-mediated cytotoxicity was defined as "natural" cytotoxicity, and the

effector cells mediating natural cytotoxicity were functionally defined as natural killer (NK) cells. The existence of NK cells has prompted a reinterpretation of both the studies of specific cytotoxicity against spontaneous human tumors (17) and the theory of immune surveillance, at least in its most restrictive interpretation, based on a predominant role of adaptive immunity and tumor antigen-specific CTLs (18, 19).

For many years a major difficulty in the study of NK cells stemmed from the fact that they were functionally defined, i.e., cells that mediate natural, non-MHC-restricted cytotoxicity. It is now known that different types of lymphocytes and other leukocytes can mediate non-MHC-restricted cytotoxicity either spontaneously or upon activation. However, most cells mediating natural cytotoxicity in humans and many other species share similar functional characteristics, and the cells appear to constitute a discrete cell subset. Unlike cytotoxic T cells, NK cells cannot be demonstrated to have clonally distributed specificity, restriction for MHC products at the target cell surface, or immunological memory. NK cells cannot as yet be formally assigned to a single lineage based on the definitive identification of a stem cell, a distinct anatomical location of maturation, or unique genotypic rearrangements. Thus, some investigators have suggested that NK cells be defined operationally, referring to any lymphoid cell from an unimmunized host mediating MHC-unrestricted cytotoxicity (20, 21).

Nevertheless, it is possible to (1) unequivocally distinguish mature NK cells from T, B, and myeloid cells; (2) distinguish NK progenitors from those of T, B, and myeloid cells; and (3) suggest that NK cells are dependent on intact bone marrow and not on thymus for their differentiation (22, 23). NK cells, therefore, represent a discrete leukocyte subset, possibly constituting a third lineage of lymphoid cells (22–25). Although the exact characterization of the NK cell subset and its possible heterogeneity still requires detailed analysis, a consensus on an operational definition of NK cells was reached at the Fifth International Workshop on Natural Killer Cells in 1988 (26). NK cells have been defined as large granular lymphocytes (LGLs) that do not express on their surface the CD3 antigen or any of the known T cell receptor chains (i.e., α, β, γ, or δ) but do express CD16 and NKH-1 (Leu-19) cell surface markers in humans and NK-1.1/NK-2.1 in mice and mediate cytolytic reactions even in the absence of MHC class I or class II expression on the target cells (26).

Certain T lymphocytes that are either $\alpha\beta^+$ or $\gamma\delta^+$ may express, particularly upon activation, a cytolytic activity that resembles that of NK cells; these T lymphocytes are more appropriately described as displaying "NK-like" activity or "non-MHC-requiring" cytolysis (26). The lymphokine-activated killer (LAK) cells, which have recently received

much attention for their possible therapeutic use (27), are interleukin-2 (IL-2)-activated lymphocytes that are NK cells or non-MHC-requiring T cells. The relative contribution of the respective cell type depends on the source of lymphocytes and conditions for activation (26).

One of the surface receptors that were identified on NK cells since their original description (11) is a low-affinity receptor for the Fc fragment of immunoglobulin G (IgG) (FcR) or CD16 antigen (28). Through their FcR (CD16), NK cells can interact with and lyse IgG antibody-coated target cells. Although antibody-dependent cell-mediated cytotoxicity (ADCC) can be mediated by a variety of cell types, including monocyte/macrophages and polymorphonucleated leukocytes (PMNs), the lymphocyte subset that mediates ADCC has been operationally defined as killer (K) cells and is identical or largely overlapping with the NK cell subset (29-33).

Although NK cells were named on the basis of the cytotoxic activity that initially served to identify them, this cell type exerts a variety of functions, including production of lymphokines, regulatory functions on the adaptive immune system and on hematopoiesis, and natural resistance against microbial infection and tumor growth (23). The cytotoxic ability of NK cells may or may not represent the most physiologically significant function of these cells *in vivo*. NK cells, together with monocyte/macrophages, PMNs, platelets, etc., are an important effector cell type of nonadaptive immunity. In mediating these functions, the activity of NK cells is regulated by a complex network of cellular and humoral interactions with cell types of the adaptive and nonadaptive immune systems, nervous system, and others. Although many tesserae of this mosaic are still incomplete or missing, this review attempts to summarize the experimental evidence pointing to NK cells as a discrete cell subset that is highly regulated in its interaction with other systems of the organism.

II. Measurement of NK Cell-Mediated Cytotoxicity

A large variety of target cell types has been used to measure NK cytotoxicity, using unseparated lymphoid preparations from human donors and experimental animals. Cultured cell lines differ greatly in their sensitivity to NK cytotoxicity and, in general, cell lines from homologous species are lysed more efficiently than are heterologous cells. Tumor-derived cell lines are often used as NK target cells (11, 14), but NK cytotoxicity can also be demonstrated against normal target cells, including normal diploid fibroblast strains (34-36). The most sensitive and widely used target cell for human NK cells is K562 (11), a cell line

derived from a patient with chronic myeloid leukemia in blastic crisis (37). These cells lack MHC class I and class II antigens and can be induced to differentiate *in vitro* to cells with myeloid, erythroid, or megakaryocytic characteristics (38, 39). The Moloney virus-induced lymphoma cell line YAC-1 is the most widely used target for the measurement of rodent NK cell cytotoxicity (40, 41).

The cytotoxic activity of NK cells can also be evaluated by the ability of these cells to lyse IgG antibody-coated target cells. In early studies ADCC activity of human lymphocytes was measured, using as target cells chicken erythrocytes (2), the Chang (HeLa) cell line (42), and T cell blasts (43) sensitized with hetero- or alloantisera. Antibody-sensitized nonnucleated erythrocytes are efficient target cells for ADCC mediated by monocytes and PMNs, but not by human NK/K cells (44). However, the use in ADCC of target cells that are sensitive to NK cell lysis in the absence of antibodies has complicated the interpretation of many studies. For this reason, the mouse mastocytoma cell line P815, which is almost completely resistant to both human and murine NK cell lysis, is now often used as the target cell for ADCC studies (32).

NK cell cytotoxicity is usually quantitated in the ^{51}Cr-release cytotoxicity assay, in which NK cell-containing cell preparations are mixed with a constant number of ^{51}Cr (sodium chromate)-labeled target cells at one or more effector-target (E : T) cell ratios, and cell lysis is evaluated, usually after 3-4 hours of incubation at 37°C, by measuring the amount of ^{51}Cr released into the supernatant fluid (45). In many studies NK cytotoxicity is expressed as the percentage of ^{51}Cr release at an arbitrarily chosen E : T ratio. However, the use of a single E : T ratio precludes a quantitative comparison of the relative cytotoxicity mediated by different donors or by the same lymphocyte preparations after different treatments. Figure 1 illustrates the great difference in relative NK activity of two donors, A and B, measured by using the percentages of ^{51}Cr release at different E : T ratios. Comparing the percentages of ^{51}Cr release at a given E : T ratio can yield the rank order of the cytotoxicity mediated by the cells of the two donors, but not a quantitative evaluation of the relative cytotoxicity. The use of several E : T ratios yields a quantitative evaluation of cytotoxicity by measuring lytic units (LU), defined as the number of effector cells required to lyse a given proportion (optimally 50%, but often 20 or 30% was used) of target cells in the assay period (3). This number can be extrapolated graphically or computed based on equations (43, 46, 47) that describe the relationship between effector cell concentration and percentage of ^{51}Cr release (Figs. 1 and 2). The use of LUs transforms a series of dose-response data to a single number (with standard errors, etc.) which is based on all of the data and which is directly proportional to NK cell lytic activity.

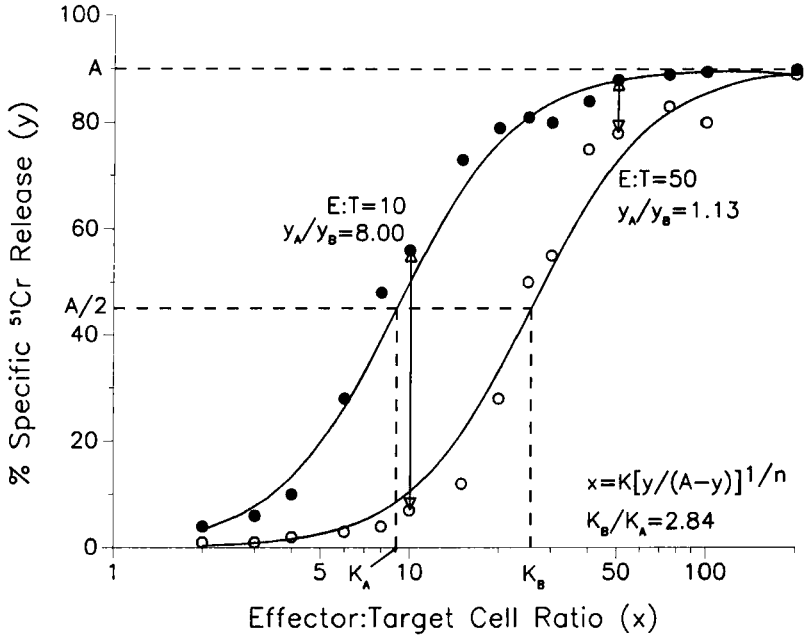


FIG. 1. Quantitation of NK cell-mediated cytotoxicity in a ^{51}Cr-release assay (4-hour incubation) using a constant number (10^4) of ^{51}Cr-labeled K562 target cells and a variable number of human PBLs as effector cells. ○, PBLs from donor A; ●, PBLs from donor B. Each symbol represents an experimental point. The relative cytotoxicity measured at the two arbitrarily chosen effector-target cell ratios (E : T) of 10 and 50 is indicated on the figure. Sigmoidal curves for the two donors were plotted using the modified von Krogh's equation (see text). When y is equal to $A/2$, K is equal to x, i.e., K × 10^4 is equal to the number of effector cells required to lyse half of the target cells (5 × 10^3) and is defined as 1 LU. LUs calculated in this way can be used to quantitate the cytotoxicity of different effector cell preparations when the slopes (n) of the different sigmoidal curves are similar. The ratio K_B/K_A represents the relative cytotoxic efficiency of the two cell preparations calculated based on all of the experimental points.

Two equations are most commonly used to reduce ^{51}Cr release dose-response data to linearity: the simple exponential fit and a modified von Krogh equation. The exponential fit equation (46-48) may be written as

$$y = A(1 - e^{-kx}) \quad (1)$$

where y is the fractional ^{51}Cr release, A is a constant equal to the asymptote of the curve, x is the E : T ratio, and k is a constant which, for curves having the same asymptote, is directly proportional to the NK cell activity. If the assay is plotted as a target survival curve, i.e., as $\ln(A - y)$ versus x, the value k is the negative of the slope of the resulting straight line (Fig. 2, bottom right). The exponential fit equation defines a sigmoidal curve on a semilog plot (e.g., Fig. 2, top).

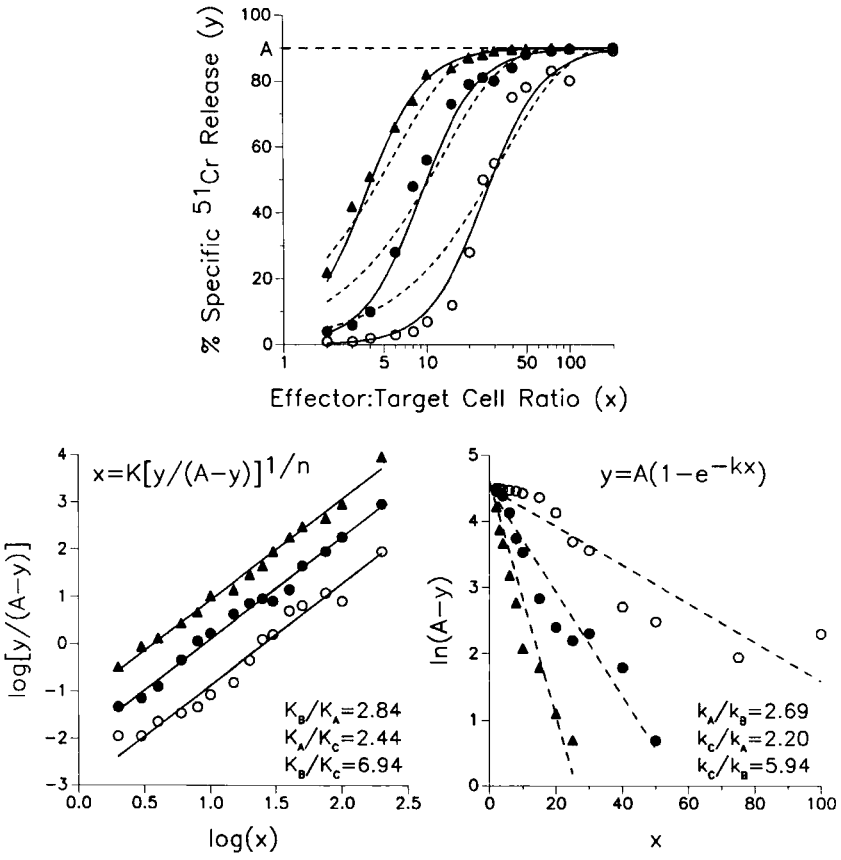


FIG. 2. Analysis of the cytotoxic activity of PBLs from donors A (●), B (○), and C (▲) using the von Krogh's equation or the exponential fit equation. The cytotoxic assay was performed as in Fig. 1. The top panel depicts the best-fit sigmoidal curves for the three donors using the modified von Krogh's equation (solid lines) or the exponential fit equation (broken lines). (Bottom left) Curves calculated according to the modified von Krogh's equation and expressed as $\log[y/(A - y)]$ versus $\log(x)$. (Bottom right) Curves calculated according to the exponential fit equation and expressed as $\ln(A - y)$ versus x. The relative cytotoxic efficiencies for the three preparations, calculated according to the two equations, are given in the bottom panels. Similar values were calculated with the two equations.

The von Krogh equation (49, 50) was originally described as an application of the Hill transformation (51) to the analysis of complement lysis and was modified for use in the analysis of ^{51}Cr-release data. Unlike complement lysis, where 100% hemolysis is obtainable, it is difficult to evaluate the maximum release of the isotope in the ^{51}Cr-release method. Complete release of incorporated ^{51}Cr is never observed, and most investigators use detergent lysis (usually 80–90% of incorporated isotope) as a measure of maximum release. However, the values observed for the maximum release upon cell-mediated cytotoxicity are usually lower than those obtained with detergent lysis and are variable among different target cells and experiments. Thus, the von Krogh equation had to be modified for analysis of ^{51}Cr-release data by the introduction of the constant A equal to the asymptote of the ^{51}Cr-release dose-response curve (43). The asymptote A has been estimated either by computer iteration (43) or experimentally (46). Like the exponential fit equation, the von Krogh equation also defines a sigmoid curve (Figs. 1 and 2, top panel), but contains a third variable which makes it possible to fit data in which there is a longer lag phase and a more abrupt rise in cytotoxicity in the exponential part of the curve. The modified von Krogh equation (43, 47) can be written as

$$y = \frac{A}{1 + (K/x)^n} \tag{2}$$

where y, x, and A are as in the exponential fit equation, n is a constant which defines the shape of the curve on the semilog plot, and K (different from the k value in the exponential fit equation) is a constant equal to x at $y = A/2$, directly proportional to NK cell activity and equal to 1 LU (50%). The modified von Krogh equation can be linearized by log transformation as

$$\log x = \log k + 1/n \log[y/(A - y)] \tag{3}$$

and a family of curves with the same n and A will therefore yield a series of parallel and straight lines when plotted $\log[y/(A - y)]$ versus $\log x$ (Fig. 2, bottom left).

Both equations have been found to result in a good correlation between the observed and calculated points (47, 48). However, the exponential fit equation is more sensitive to changes in the A value in calculating the LU. The von Krogh equation yields a better fit under these conditions, because it contains the slope of the line, $1/n$, as a third variable. However, because of this variable, a serious source of error is introduced in the LU calculation, and results of different effector cell preparations or of different target cells can be compared only when the slopes of the different curves are not significantly different.

The use of the cytotoxic assay to compare the NK cell activity of different normal donors or patients is complicated by a large variability

of activity among normal donors and by day-to-day variation in sensitivity of the assay system. In sequential studies normalization of the assay is necessary in order to compare the results obtained in different experiments. The work of Pross and collaborators (48, 52-55), who studied this problem in detail, has generated several suggestions for the normalization of the NK cell assay. The rank of cytotoxic activity mediated by lymphocytes from normal donors remains relatively constant over an extended period, in the absence of situations such as infection or drug treatment that alter NK cell activity (52, 56). It is therefore possible to normalize the cytotoxic assay by using in each experiment a group of control donors with similar average activity, but not necessarily the same control donors in each experiment. However, because the repeated use of fresh normal controls is frequently impractical, cryopreserved lymphocytes are often used. NK cell functions are usually markedly reduced after cryopreservation (57), but are almost completely recovered if the lymphocytes are incubated for a few hours at 37°C after thawing (53). Although cryopreservation may reduce the absolute cytotoxic activity, the relative cytotoxicity of lymphocytes obtained from different donors is maintained, making the use of cryopreserved lymphocytes for normalization of the cytotoxic assay possible (53). Normalization in the cytotoxic assay is absolutely necessary in order to compare results within experiments or among different laboratories. Unfortunately, many of the published analyses of NK cell activity in patients completely lack any normalization.

Several factors that can affect *in vivo* or *in vitro* NK cell activity must be considered in analyses of NK cell cytotoxic activity of lymphocytes from patients. NK cell activity tends to increase with donor age and is, on average, higher in male than in female donors, making it important to use a control group that is age and gender matched (52). Alcohol, smoking, various common drugs (such as salicylates), stress, and concurrent diseases (such as infections) may also alter NK cell activity *in vivo*. *In vitro*, the presence of monocytes and PMNs can suppress NK cell cytotoxic activity, whereas the presence of erythrocytes in the assay determines a dose-dependent enhancement of cytotoxicity (58).

Other NK cell cytotoxic assays allow a direct microscopic observation of the effector-target cell interaction. In the single-cell cytotoxic assay in agarose, effector and target cells are allowed to form conjugates in a pellet for a few minutes, and the conjugates are then immobilized in smears of semisolid medium (agarose) (59, 60). The NK cells are prevented from recycling by the agarose. The smears, on petri dishes or on microscope slides, are incubated at 37°C for various periods, and the dead cells are evaluated by dye exclusion, using trypan blue. The slides can then be fixed and the conjugates and lytic conjugates can be

counted. Different investigators have reported 15-40% human peripheral blood lymphocytes (PBLs) forming conjugates with K562 cells (48, 61-63). Although a large proportion of NK cells [up to 100% after interferon (IFN) stimulation] bind to target cells, not all conjugate-forming cells in human peripheral blood are NK cells. This has been clearly shown in several studies in which the phenotype of binding cells has been analyzed (63-66). As evaluated in the single-cell assay, the frequency of lytic NK cells in human peripheral blood has been reported to be 1-5% (61, 63). Combined use of the single-cell cytotoxic assay in agarose and estimation of the maximum NK cell cytotoxic potential by ^{51}Cr release to study recycling of effector cells indicated that, on average, an NK cell can lyse 2.3 target cells (62, 67).

Although laborious and difficult to quantitate, the single-cell assay allows an approximation of the number of active NK cells in cell preparation, and it has been extremely useful to study the mechanisms of cytotoxicity and their alteration in patients or upon *in vivo* or *in vitro* drug treatments. However, caution should be exercised in interpreting data, especially those concerning NK cell recycling that are based on the assumption that the single-cell assay has 100% efficiency in allowing conjugation and killing by active NK cells.

Under appropriate experimental conditions, cell-mediated cytotoxicity can be analyzed in a manner analogous to enzyme-catalyzed reactions. Initial studies of the kinetics of cellular cytotoxicity reactions generally applied the equation for simple enzyme kinetics originally developed by Michaelis and Menten (68-70). However, cellular cytotoxicity reactions do not follow simple Michaelian kinetics. The experimentally determined apparent Michaelis constant (K_m^{app}) varies in proportion to the number of lymphocytes present in the assay system (71). Because of the differences between enzyme-catalyzed reactions and cellular cytotoxicity reactions, more complex models were developed. Merrill (72) developed more general equations that took into account the possibility of noncytotoxic lymphocytes binding to target cells and inhibiting cytotoxicity. In this model V_{max}, the maximum velocity for a natural cytotoxicity reaction, is expressed as

$$V_{max} = k_2 \alpha f [L] \tag{4}$$

where $[L]$ is the lymphocyte concentration, f is the fraction of target-binding lymphocytes, α is the fraction of cytolytically active target-binding lymphocytes, and k_2 is the rate constant for target cell lysis. The expression of K_m^{app} that results from this model is very complex and takes into consideration the rate constants for programming for lysis (see Section V) and for target cell disintegration, the dissociation constant for target conjugates of nonlytic lymphocytes, and the fractions of lymphocytes that bind target cells and lyse target cells (72). However, for cytotoxicity

mediated by human NK cells, Callewaert *et al.* (73, 74) determined that programming for lysis is the rate-limiting step and the value of K_m^{app} is directly related to the frequency of target-binding cells within the lymphocyte population. K_m^{app} can be approximated by the expression

$$K_m^{app} = f[L](K_m/K_I) \quad (5)$$

where K_m is the standard Michaelis-Menten constant and K_I is the dissociation constant for target-binding nonlytic lymphocytes.

V_{max} is a useful quantitative measure of the overall cytotoxic activity of a lymphocyte preparation. V_{max} values increase linearly with an increasing number of lymphocytes in the assay and are useful for the quantitative comparison of the relative cytotoxic activity of different lymphocyte preparations. V_{max} and LU values yield comparable estimates of relative cytotoxic activity (75). The physical significance of K_m^{app} is more difficult to interpret. For NK cell-mediated cytotoxicity, K_m^{app} is not constant but varies with the concentrations of lymphocytes tested, and it is approximately equal to the concentration of lytic lymphocytes (71). K_m^{app} therefore allows for the simultaneous determination both of the frequency of NK effector cells, according to the relationship

$$\% \text{ NK} = K_m^{app}/[L] \times 100 \quad (6)$$

and of the activity of NK effector cells, by determining the rate constant for target cells according to the relationship

$$k_2 = V_{max}/K_m^{app} \quad (7)$$

The initial rate of K562 cytolysis by human NK cells is maintained for 1–3 hours, followed by a stable plateau of cytotoxicity values (Fig. 3), reflecting the inability of NK cells to lyse additional target cells unless stimulated with IFN or IL-2 (76, 77). These results suggest that, although NK cells are able to lyse more than one target cell, their recycling ability, unlike that of CTLs, is extremely modest (76). The kinetics analysis of NK cell cytotoxicity is further complicated when target cell preparations are used that stimulate production of lymphokines, affecting NK cell cytotoxicity during the assay. For example, production of IFN-α or -γ, by NK cells or other cell types present in the lymphocyte preparation, is observed when target cells are sensitized with IgG antibodies (78) or infected with viruses (79, 80) or mycoplasmas (81, 82). A typical example is the lysis of virus-infected target cells in which V_{max} significantly increases after 4–6 hours of culture, because of IFN production or other stimuli for NK cells (56) (Fig. 3).

III. Phenotypic and Genotypic Characteristics of NK Cells

A. Identification of NK Cells

Identification of NK cells based solely on their ability to mediate spontaneous and antibody-dependent cytotoxicity, a function shared with other cell types, such as monocyte/macrophages and activated T cells,

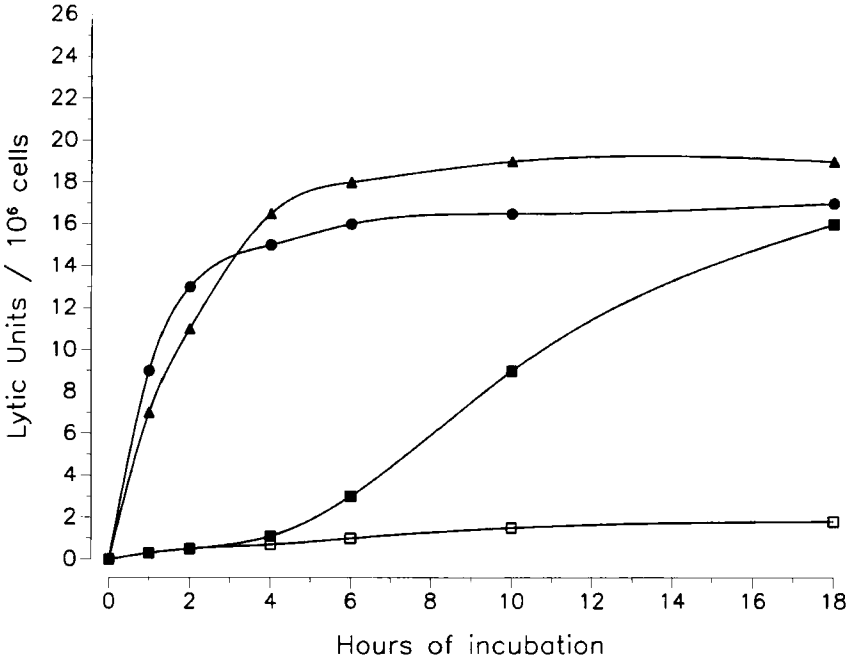


FIG. 3. Kinetics of NK cell-mediated cytotoxicity. PBLs from one donor were tested against 10^4 K562 cells (●—●), rabbit IgG-coated P815 cells (▲—▲), human fibroblasts (□—□), or human fibroblasts infected with the HK strain of influenza virus (■—■) in a ^{51}Cr-release cytotoxicity assay. Cytotoxicity was evaluated at different times and LUs were calculated from various effector-target cell ratios using the modified von Krogh's equation.

has represented a major limitation in the analysis of NK cells. One of the most significant contributions to the study of NK cells has been their identification as a relatively homogeneous cell type on the basis of physical and phenotypic characteristics and their LGL morphology (35). Human NK/K cells were originally described as nonadherent, nonphagocytic, FcγR-positive cells with lymphoid morphology. Although velocity sedimentation experiments demonstrated that human K cells were larger than the bulk of T lymphocytes (44), it was not until Saksela, Timonen, and collaborators (35, 83, 84) analyzed cytotoxic effector cells adsorbed-eluted from both fibroblast and cell line target cells that NK cells were identified as LGLs, i.e., large lymphocytes with a high cytoplasm-nuclear ratio and few discrete azurophilic granules. A separation technique involving a discontinuous Percoll gradient has been widely used for the enrichment of LGLs based on their light buoyant density (85). This technique has contributed much to the progress of studies of NK cells, allowing investigations utilizing semipurified preparations of NK cells. Such

preparations have been used for the analysis of surface phenotype and morphology as well as functional characteristics of NK cells (86–90). However, the use of these semipurified preparations has also generated considerable artifact and confusion, due mostly to disregard of the following facts: (1) LGL morphology is not unique to NK cells, and not all NK cells may have typical LGL morphology at all times during differentiation and functional activation (91); (2) light-density Percoll fractions, although enriched for NK cells/LGLs, also contain monocytes, dendritic accessory cells, human leukocyte antigen (HLA)-DR^+ IFN-α-producing cells, T cell blasts, and memory T cells (92, 93), and even some of the most careful purification procedures could not completely eliminate all of these contaminant cell types; (3) whereas light-density Percoll fractions are enriched for accessory cells, enriched T cell preparations from high-density fractions are completely devoid of accessory cells; thus, some of the reported differential activity of NK and T cells might rest in the presence or absence of accessory cells in the cell preparations used.

The use of monoclonal antibodies to cell surface markers has greatly contributed to the progress in the identification of the NK cell subset. These studies are now being extended, with the use of molecular probes, to assay for gene transcript expression and genotypic organization of NK cells. Various methods of identification or purification of NK cells have been used for the phenotypic analysis of NK cells using monoclonal antibodies and molecular probes.

1. Elimination of antibody-positive cells with antibody and complement, or separation of cells by positive or negative selection using fluorescence-activated cell sorting or indirect antiglobulin rosetting methods, followed by analysis of the cytotoxic activity of the different cell preparations (66, 94–96), has been very successful, although in some cases not useful, in distinguishing between effector and accessory cells. A serious difficulty in the studies of positive selection stems from the possibility, now demonstrated for several cell surface markers, that the reaction of antibodies with surface receptors on NK cells alters the cytotoxicity and other functions of NK cells.

2. Analysis of surface markers of enriched preparations of LGLs from Percoll gradients has generated some confusion due to the presence of contaminant cells in the LGL preparations; however, they have also contributed to the identification of these contaminants and have provided means, using negative selection with appropriate monoclonal antibodies, to eliminate them (89).

3. Combined use of monoclonal antibody analysis, by fluorescence or complement cytotoxicity, and single-cell assay in semisolid medium has been used for a direct and accurate phenotypic analysis of both target

cell-binding lymphocytes and lytic effector NK cells (64, 66). Although powerful, these methods are technically difficult, laborious, and tedious to perform and have not been widely used.

4. Isolation of lymphocyte clones with NK cell activity, dependent on IL-2 for growth and usually with a limited *in vitro* life span, has been recently reviewed in another volume of this series (20). The availability of NK cell clones provides a unique opportunity to study NK cell functions and characteristics using homogeneous cell preparations. However, the ability to mediate non-MHC-restricted cytotoxicity is not a unique property of NK cells, and some T cells, especially after IL-2 activation, can also mediate non-MHC-restricted cytotoxicity (20, 97-99). Indeed, most of the NK clones described in early studies were of T cell origin (20). As in the case of Percoll separation of LGLs, the method of NK cell cloning has allowed some of the most significant progress in the study of NK cells, but has also generated artifactual information that underlies much of the current controversy and confusion in the NK cell field.

5. With bulk expansion of NK cells in short-term cultures using different methods (100, 101), large numbers of nearly pure NK cells can be obtained and used in biochemical and molecular studies that would be impossible to perform on the limited numbers of NK cells obtainable from fresh peripheral blood or spleen. However, as in the case of NK cell clones, the use of these preparations carries the possibility of selective expansion of NK cell subsets and the use of *in vitro* activated cells with functional and phenotypic characteristics different from those of resting NK cells obtained *ex vivo*.

6. The rat leukemia RNK-16 cell line, which has spontaneous cytolytic ability against YAC-1 cells and characteristics of NK cells (102), has been used for biochemical and functional analyses of NK cells and their cytotoxic mechanism. Human leukemias, chronic or acute, with expansion of cells with NK cell characteristics are also known, although rare, and have been used in some studies for the analysis of NK cell characteristics (103).

Except for the artifacts due to the use of contaminated or non-characterized NK cell preparations, the results obtained using all of these different approaches for the identification of NK cells have been, in general, consistent and serve to identify NK cells as a discrete lymphocyte subset with phenotypic and genotypic characteristics different from those of T and B cells.

B. Surface Phenotype of Human NK Cells

Early studies on human NK cells showed that virtually all of these cells express FcγR and about 50% of them form low-affinity rosettes

with sheep erythrocytes at 4°C, but, unlike T cells, only a small proportion form high-affinity rosettes at 29°C (16, 104). The presence of complement receptors (CRs) on NK cells has been a controversial issue (11, 105-107). Most NK cells are now known to express the receptor for C3bi (CR3 or CD11b) but not those for either C3b (CR1 or CD35) or C3d (CR2 or CD21) (108-111). The use of monoclonal antibodies has revealed no surface antigen unique to NK cells, but rather a unique combination of antigens, each shared with other cell types, mainly T cells and myelomonocytic cells. Figure 4 summarizes the antigenic phenotype

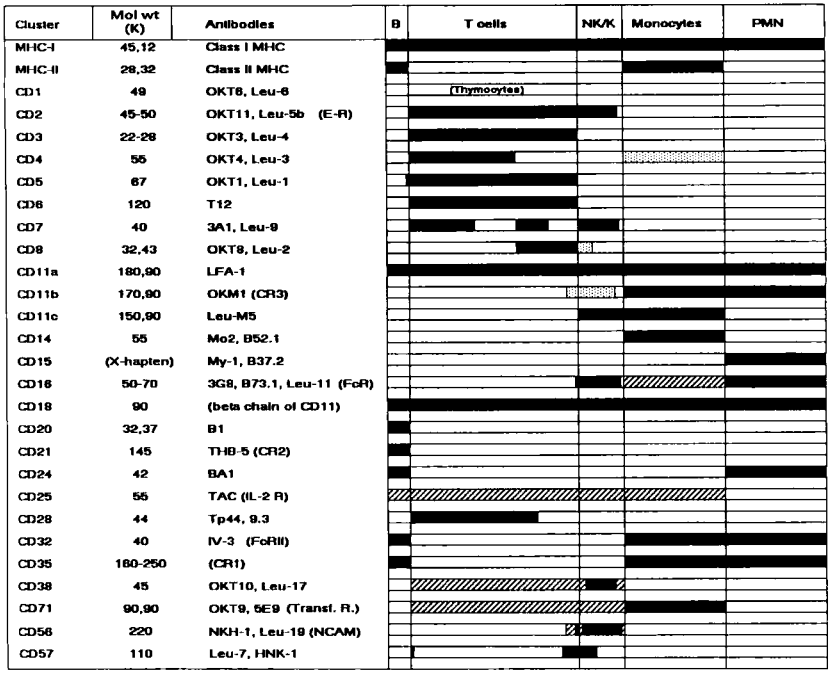


FIG. 4. Surface markers of human NK/K cells as compared to B cells, T cells, monocytes, and polymorphonucleated neutrophilic cells (PMNs). The antigens are designated according to the clusters of differentiation (CD) defined for the leukocyte differentiation antigens. The molecular weights of the precipitated molecules (reduced form) and the prototype antibodies used for their identification are also indicated. The length of the filled bars within each cell population indicates the approximate proportion of cells expressing the antigens. However, the position of the bars within each cell population may not always be representative of the overlapping or exclusive expression of the antigens on different subsets. Solid bars, positive cells; stippled bars, low-density positive cells; hatched bars, activated cells only.

of NK cells in comparison to other leukocyte populations. Often, these cell surface markers are not present on all NK cells, suggesting some heterogeneity within the NK cell population. In general, human NK cells lack cell surface marker characterisrics of B cells, with the exception of the recently described CB02 antigen, present on B cells and a subset of NK cells (112). Most investigators agree that resting human NK cells do not significantly express class II MHC antigens (95, 113, 114), although Brooks and Moore (115) have reported the expression of HLA-DR, -DP, and -DQ antigens on a subset of NK cells.

1. FcR (CD16) Antigen

Various types of FcγR have been identified on human hematopoietic cells. Monocytes and macrophages express at least two types of FcγR: a high-affinity ($K_a \sim 10^8/M$) receptor (p72 or FcRI) able to bind monomeric IgG, and a low-affinity ($K_a \sim 10^6/M$) receptor (gp40, FcRII, or CDw32), also expressed on PMNs and B cells. PMNs also express a third type of FcγR (CD16 antigen) and, when activated by immune IFN (IFN-γ), FcRI. CD16 FcR is also expressed on the large majority of NK cells and on tissue macrophages as well as on monocyte-derived macrophages. B cells express only CDw32 FcR.

CD16 FcR is a low-affinity receptor that binds IgG in immune complexes with soluble or insoluble (e.g., antibody-coated cells) antigen but does not bind monomeric IgG. Several monoclonal antibodies produced against CD16 FcR (28, 95, 116–118) bind to few different antigenic determinants on the CD16 molecule and, as discussed below, might have different cellular specificity. During differentiation of PMNs, CD16 antigen appears at a late stage of myeloid differentiation in the bone marrow (metamyelocytes or later). In the peripheral blood, CD16 is expressed on virtually all neutrophils, but only on eosinophils with a more mature morphology. Basophils do not express CD16 FcR. Circulating monocytes express little, if any, CD16 FcR, but *in vitro* cultured monocytes express it at high density. Due to the limited information on NK cell differentiation, it remains unknown when CD16 FcR is first expressed on these cells.

The first anti-CD16 antibody, 3G8, was shown to react with PMNs and macrophages (116). Another anti-CD16 monoclonal antibody, B73.1 (95), reacts with the large majority of human NK cells. CD16$^+$ lymphocytes contain virtually all of the lymphocytes able to mediate spontaneous cytotoxicity. Although not present exclusively on NK cells, the CD16 antigen still represents the best marker to identify and purify NK cells among peripheral blood mononuclear cells. Unlike 3G8, antibody B73.1 reacts with PMNs of only 50% of donors; both antibodies react with NK cells from all donors. Other antibodies that cross-compete with B73.1

but not with 3G8 for binding to NK cells also react with PMNs from only a proportion of donors. 3G8 and B73.1 (and other antibodies, such as anti-Leu-11a/b, CLB FcR-gran 1, and VEP-13) react with at least two different determinants on the CD16 molecule (28). Although the significance of this differential cellular reactivity of anti-CD16 antibodies to different determinants of the molecule is unknown, this observation points to a possible heterogeneity of the CD16 molecule on different cell types. The anti-CD16 antibody, CLB-gran 11, never reacts with NK cells and detects only PMNs of donors carrying the allele NA1 (phenotypic frequency, 46%) of the neutrophil-specific NA antigen biallelic system (119). These results show that PMN CD16 FcR, but not NK cell CD16 FcR, carries the NA antigenic determinants.

The antibody AB8.28 (120, 121) was originally described as specific for NK cell FcγR, based on its ability to block rosette formation with antibody-coated erythrocytes. However the antigen recognized by this antibody shows different molecular characteristics from those of CD16 antigen, is still present on the cells when the CD16 FcR is down-modulated by antibodies or immune complexes (122), and rapidly disappears from NK cells in culture when CD16 FcR is strongly expressed. The exact nature of the AB8.28 antigen remains to be established.

The CD16 molecules precipitated from the membrane of PMNs or NK cells appear on sodium dodecyl sulfate (SDS) gels as a broad band corresponding to a molecular weight between 50,000 and 70,000 (95, 116). The molecules are highly glycosylated and, after treatment with *N*-glycanase, resolve in small products as bands migrating at 23–28 and 32–36 kDa from PMNs and NK cells, respectively (123–126). From monocyte-derived macrophages, the anti-CD16 antibodies precipitate a 53 kDa species minimally altered, if at all, by treatment with *N*-glycanase (123). These results suggest that the CD16 antigen is expressed on different cell types on molecules with different levels of glycosylation and with a polypeptide backbone of different lengths.

Recently, a complementary DNA clone encoding CD16 determinants was isolated that gave rise to IgG-binding molecules with affinity and specificity expected for CD16 in transfected COS cells (124). The cDNA was isolated from an expression library of human placenta, and therefore the exact cellular origin is unknown.

CD16 cDNA-transfected COS cells bound human IgG_3 and IgG_1 and mouse IgG_{2a} and IgG_3 with a K_a of $\sim 10^6/M$ and murine IgG_1 with lower affinity, but did not bind human IgG_2 and IgG_4 or murine IgG_{2b} (124).

The cloned CD16 cDNA sequence spans 888 nucleotides and encodes a predicted peptide of 233 residues. The first 18 residues have the typical feature of a secretory signal sequence. The signal peptide is followed

by two Ig-related segments with two intrachain disulfide bonds and significant homology to members of the constant-region C-2 set of the Ig superfamily. The predicted molecular mass of the expected polypeptide (~25 kDa) is consistent with that observed for CD16 molecules precipitated from PMNs and treated with *N*-glycanase. The polypeptide sequence ends with a short hydrophobic domain (residues 200-220), followed by four hydrophilic residues. A similar structure and hydropathicity profile is shared by membrane proteins bearing glycosyl phosphatidylinositol phospholipid (GPI-PL)-linked carboxy termini. Various groups (124, 127, 128) have provided evidence that the CD16 molecules are linked to the cell membrane through GPI-PL: (1) CD16 molecules are removed from the cell membrane and released into the supernatant when PMNs or CD16-transfected COS cells are treated with GPI-specific phospholipase C (GPI-PLC), and (2) PMNs from patients with paroxysmal nocturnal hemoglobinuria (PNH), an acquired abnormality affecting GPI tail biosynthesis or attachment, lack expression of CD16 FcR. However, the possibility remains that CD16 FcR in cell types other than PMNs (such as NK cells and macrophages) is at least in part a transmembrane molecule, as suggested by (1) heterogeneity in CD16 polypeptide molecular mass in different cell types, (2) normal expression of CD16 FcR on NK cells and cultured monocytes from PNH patients (127-129), and (3) an inability of GPI-PLC to remove CD16 FcR from human NK cells (124, 126). Both PMNs and NK cells spontaneously shed CD16 antigen in the absence of GPI-PLC treatment. After digestion with *N*-glycanase, the CD16 antigen shed from NK cells resolves in SDS gels in 23-28 kDa smaller fragments identical to those precipitated from both PMN supernatant and cells (126). Thus, the CD16 antigen on NK cells and PMNs might undergo spontaneous proteolytic cleavage at the same position, but, unlike in PMNs, the cleaved antigen from NK cells fails to remain on the membrane as a GPI-linked molecule and is released into the supernatant.

The CD16 sequence shares highest homology with the α gene of the murine FcRII, followed by the human CDw32 and the β gene of murine FcRII. Interestingly, murine NK cells express RNA transcripts of the α gene but not of the β gene of FcRII (130). The homology between CD16 FcR and murine FcRIIα genes extends through the putative transmembrane portion (17 identical nucleotides of 21 in that domain), but the α transcript, unlike the CD16 transcript, presents a long intracytoplasmatic domain, suggesting that the murine FcRIIα genes are transmembrane proteins. Analysis of FcγRIII (CD16) transcripts isolated from PMN and NK cells of single donors revealed multiple single nucleotide differences between these respective sequences (126). One of

these differences converts an in-frame UGA termination codon to a CGA codon, resulting in an extended open reading frame encoding 21 additional amino acids (126). The CD16 FcR transcripts in NK cells therefore encode a 25-amino acid intracytoplasmatic domain that is highly homologous to that of murine FcRIIα (126). Recently, two nearly identical, linked genes have been cloned for Fcγ RIII (CD16). These genes are transcribed in a cell-type specific fashion to generate the alternatively anchored forms of this receptor (126). The close analogy between CD16 FcR and the murine FcRIIα indicates that CD16 is expressed on a molecule that represents the human equivalent of murine FcRIIα.

The functional relevance of the structural differences between CD16 FcR on NK cells and PMNs is indicated by the fact that NK cells, but not PMNs, are able to lyse anti-CD16 antibody-producing hybrid cells, indicating that CD16 antigen on NK cells functions as a signal-transducing structure in ADCC (125, 131). On the other hand, PMNs lyse anti-CDw32 FcRII-producing cells, indicating that in these cell types CDw32 FcRII, but not CD16 FcR, functions as signal-transducing structures in ADCC (131).

The surface expression of CD16 on human NK cells is highly regulated. Incubation of NK cells with anti-CD16 antibodies or immune complexes determines a rapid disappearance of the CD16 antigen from the cell surface (132). Treatment of NK cells with phorbol diesters also induces complete down-modulation of CD16 antigen expression in a few minutes (133). When anti-CD16 antibodies are cross-linked by a second anti-mouse Ig antibody on the NK cell surface, the CD16-antibody complex is internalized, as demonstrated by the disappearance of the antibody from the surface and by its release into the supernatant in the form of small proteolytic fragments (132, 134). However, CD16 antigen is spontaneously released from NK cells and PMNs, and release of CD16 from PMNs is increased by activation with chemotactic peptides (127). It is therefore possible that shedding of CD16 antigen plays some role in the down-modulation observed with antibodies and with phorbol diesters. In most healthy donors the CD16 antigen is expressed on an insignificant proportion of $CD3^+$ T cells. However, the presence of approximately 5–10% $CD3^+$, $CD16^+$ T cells has been described in two of 50 donors tested (135). These T cells express CD16 antigen at a much lower density than do $CD3^-$, $CD16^+$ NK cells. $CD3^+$ clones expressing CD16 antigen have also been described (136), and $CD3^+$, $CD16^+$ is a common phenotype of the cells from LGL lymphocytosis patients, as discussed later in this review.

CD16 FcR is the receptor used by NK cells for recognition of antibody-coated target cells, and in general only $CD16^+$ clones have ADCC activity. It was proposed that $Fc\gamma R^+$ and $Fc\gamma R^-$ subsets of NK cells mediate cytotoxicity against K562 cells and the Burkitt's lymphoma cell line, Daudi, respectively (137). However, these data have not been

confirmed using anti-CD16 antibodies. CD16 antigen is expressed on more than 95% of the peripheral blood cells with cytotoxic activity for both K562 and Daudi target cells (95).

2. *NKH-1/Leu-19 Antigen*

A series of antibodies was produced that reacts with most NK cells and precipitates a molecule of molecular weight 200,000-220,000, often referred to as NKH-1 or Leu-19 antigen. The first antibody described, N901, was derived from mice immunized with cells from a chronic myeloid leukemia patient in blastic crisis (138). In addition to reacting with NK cells, antibody N901 recognizes an antigen expressed at high density on the immature myeloid cell line KG1a and on the majority of cells from some patients with acute myeloid leukemia (138). N901 also reacts with neurons, neuroblastoma cell lines, and human teratocarcinoma cells, especially after induced differentiation to neural cells (139; P. Andrews, personal communication). The NKH-1/Leu-19 antigen has been shown recently to be expressing the neural adhesion protein N-CAM (L. Lanier, personal communication). Two other antibodies, NKH-1A (140) and anti-Leu-19 (141), were shown to react with NK cells with the same specificity as N901 and to precipitate a protein of 200 kDa. Binding competition among different antibodies with this specificity is not usually observed, suggesting the existence of several antigenic sites on the molecule. The NKH-1/Leu-19 antigen is expressed at very low density on peripheral blood NK cells, but its density increases significantly following *in vitro* stimulation and growth of NK cells (100). The subset of PBLs expressing the NKH-1/Leu-19 antigen (on average, 15% of lymphocytes and 90% of LGLs) almost completely overlaps with that expressing the CD16 antigen (141). The $CD16^-$, $NKH\text{-}1^+$ cells, representing 2-3% of PBLs, can be subdivided into two subsets based on expression of the CD3 antigens (141). $CD3^-$, $NKH\text{-}1^+$, $CD16^-$ cells are probably NK cells that do not express the CD16 antigens because of differentiation or activation state. $CD3^+$, $NKH\text{-}1^+$, mostly $CD16^-$, cells represent a minor subset of T cells with low but significant non-MHC-restricted ability (141). Expansion *in vitro* of $CD3^+$, $NKH\text{-}1^+$ lymphocytes generates a high proportion of clones expressing the T cell receptor for antigen (TCR)-associated clonotype NKTa, which mediate non-MHC-restricted cytotoxicity by recognizing, via the TCR, the antigen TNK TAR detected by antibody 4F2 on proliferating target cells (142-144). NKH-1/Leu-19 antigen is almost invariably expressed on clones with non-MHC-restricted specificity, both $CD3^-$ and $CD3^+$ (20). However, the presence of NKH-1/Leu-19 antigen on some clones without cytotoxic activity excludes an association between the antigen and cytotoxicity (145).

Another monoclonal antibody, anti-NKH-2, also shows selective reactivity for LGLs (140) and precipitates a molecule of 60 kDa, distinct from NKH-1. About 7% of PBLs express NKH-2 antigens and partially overlap

with the NKH-1$^+$, subsets. The NKH-1$^+$, NKH-2$^-$ cells in peripheral blood appear to have higher cytotoxic activity than do NKH-1$^+$, NKH-2$^+$ cells, although highly cytotoxic clones of either phenotype have been described (20, 140).

3. HNK-1/Leu-7 Antigen

The reactivity of antibody HNK-1 (anti-Leu-7), originally described as NK cell specific (146), is complex. This IgM antibody precipitates a 110-kDa antigen from PBLs and reacts with 30–70% of peripheral blood NK cells, with variability among donors (95, 96). Unlike the observation for CD16 and NKH-1/Leu-19 antigens, there is no correlation between the percentage of PBLs positive for HNK-1/Leu-7 antigen and NK cell cytotoxicity (95). The expression of HNK-1/Leu-7 is rapidly lost *in vitro*, and neither bulk cultures nor clones of NK cells express it (20, 100, 147). Cord blood NK cells, which normally express CD16 antigen and have reduced but significant NK cell activity, do not express HNK-1/Leu-7 antigen (95, 148). In addition to its reactivity with some NK cells, HNK-1 reacts with a variable proportion of CD3$^+$, CD8$^+$ and sometimes HLA-DR$^+$ T cells (149) and also with a rare population of CD4$^+$ T cells that is expanded in various pathological conditions (150–152). These CD4$^+$, HNK-1/Leu-7$^+$ cells are present in physiological conditions in germinal centers of lymphoid tissue and are granular lymphocytes with a lower ability to produce IL-2 and B cell-stimulating factor than HNK-1/Leu-7$^-$, CD4$^+$ helper T cells (153, 154). A morphology of LGLs or at least the presence of granules seems to characterize most HNK-1/Leu-7$^+$ lymphocytes with both T and NK cell markers (149).

The CD3$^+$, CD8$^+$, HNK-1$^+$ cells, which phenotypically resemble T cells but have a reduced response to mitogenic or allogeneic stimulation and are slightly larger and more granular than most T cells, have been proposed as the NK precursors (149, 155). However, transformation or maturation from one cell type to the other has never been demonstrated, and the presence of TCR gene rearrangement in CD3$^+$, HNK-1$^+$ but not in CD3$^-$, HNK-1$^+$ has definitively negated this hypothesis. Based on the present understanding of the specificity of HNK-1 antibody, most of the conclusions on NK cell biology and functions reached using this antibody as the only probe should be rejected and HNK-1 should not be used as an NK cell marker.

Four subsets of PBLs have been distinguished on the basis of reactivity of PBLs with HNK-1 and anti-CD16 antibody (96, 149): CD3$^-$, CD16$^+$, HNK-1$^-$ NK cells, with the highest cytotoxic activity; CD3$^-$, CD16$^+$, HNK-1$^+$ NK cells, with intermediate cytotoxic activity; CD3$^+$, CD16$^-$, HNK-1$^+$ T cells, with low (or null) cytotoxic activity; and CD3$^+$,

CD16$^-$, HNK-1$^-$ small T cells, with no cytotoxic activity. Interestingly, the T cells that can be induced to become cytotoxic by treatment with IL-2 are mostly included in the CD3$^+$ HNK-1$^+$ subset (156). Moreover, CD3$^+$, CD4$^+$, HNK-1$^+$ PBLs bind but do not lyse NK cell-sensitive target cells (152).

Several cell types other than lymphocytes react with HNK-1/Leu-7 antibody. The antigen recognized by the antibody is present on myelin-associated glycoprotein (MAG) (157, 158), and several anti-MAG antibodies have the same specificity on lymphocytes as the HNK-1 antibody (159–162). Antibody HNK-1 reacts with peripheral nerves, spinal cord, small-cell carcinoma and adenocarcinoma of the lung, endocrine cells of the fetal bronchus, other neuroendocrine cells, and hypertrophic and malignant prostatic epithelium (163–168). HNK-1 also reacts with neural cell adhesion molecules (169). However, the possibility that antibody HNK-1 recognizes on NK cells an adhesion molecule involved in target cell recognition seems quite unlikely, since HNK-1$^-$ NK cells are more cytotoxic than are HNK-1$^+$ NK cells and expression of the antigen is rapidly lost on highly cytotoxic NK cell cultures and clones (20, 100). The reactivity of many different glycoproteins and cell types with HNK-1 antibody is explained by shared carbohydrate moieties (170, 171). The carbohydrate recognized by HNK-1 is present on both glycoproteins and glycolipids (170). The glycolipid recognized is an unusual glucuronic acid-containing sulfated glycosphingolipid with five sugars but without sialic acid (171).

Monoclonal IgM antibodies with the same reactivity as HNK-1 are present in the serum of patients with peripheral polyneuropathy, a benign chronic demyelinating disease of older patients (172–174). Although the monoclonal IgM from patients shows binding competition with HNK-1, suggesting an identical antigenic specificity, binding affinity is very low compared to HNK-1 and binding can be demonstrated at 4°C but not at 37°C (159). It is therefore unlikely that the human monoclonal antibodies bind *in vivo* to circulating NK cells. Neuropathy patients with monoclonal paraproteinemia usually have a normal number and activity of NK cells (175, 176), although a decreased number of HNK-1$^+$ cells was reported in three patients (177).

4. CD11/CD18 Antigens and Myelomonocytic Antigens

CD11/CD18 is a family of three molecules composed of a common β subunit (CD18, 95 kDa) and different α subunits: CD11a or LFA-1, CD11b or CR3, and CD11c or p150 (178). All three molecules are expressed on human NK cells (179). CD11a or LFA-1 is expressed on all lymphocytes, whereas CD11b and CD11c tend to be expressed preferentially on NK

cells/LGLs (110, 179). CD11b is strongly expressed on PMNs and monocytes (180). The reactivity of anti-CD11b antibody OKM1 with NK cells was first reported as evidence for the myeloid nature of NK cells (94, 110). However, CD11b is present at low intensity in the majority of, but not all, NK cells, is expressed on some T cells, and rapidly disappears from NK cells maintained in culture (100)

Two monoclonal antibodies, H25 and H366 (181, 182), generated against the human T cell line HSB-2 and precipitating polypeptide chains of 96 and 53 kDa, respectively, have been described to react with all NK cells and with few other PBLs. Antibodies H25 and H366 react with monocytes, myeloid and erythroid precursor cells, and myeloid blasts and promyelocytes, but not with more mature myeloid cells (181, 182).

With the exception of CD11b, CD16, NKH-1, H25, and H366, none of a series of other antigens present on myelomonocytic cells at various stages of differentiation is expressed on NK cells (183, 184).

5. T Cell-Associated Antigens and TCR

Human NK cells do not express the 69-kDa CD5 membrane antigen present on all T cells (95, 183, 185), although anti-CD5 antibodies react with a cytoplasmic antigen on permeabilized NK cells (186). CD4 antigen is not expressed on NK cells, whereas 30–50% of NK cells express the CD8 antigen at characteristic low density (66). The CD8 antigen precipitated by anti-CD8 antibodies from NK cells appears identical on SDS gels to that precipitated from T cells (132). CD8 antigen expression is maintained in bulk cultures of NK cells (100), but $CD3^-$ $CD8^+$ NK clones are rare (20). $CD8^+$ and $CD8^-$ NK cells have similar cytotoxic activity and no other different functional abilities have been identified in the two subsets (66). The CD7 and the D44 (187) antigens are also expressed on NK cells.

A proportion ($\sim$50%) of NK cells express a low-affinity receptor for sheep erythrocytes (E-R), forming rosettes at 4°C but not at 29°C. However, $\sim$90% of NK cells react with anti-CD2 antibodies, which detect SRBCs. The expression of CD2 is more heterogeneous on NK cells than on T cells, and there is no correlation between CD2 expression and cytotoxic ability of NK cells.

Antibody NK9 (188, 189) against a distinct sialylated antigen of the T200 family was described to react specifically with NK cells, CTL precursor cells and both allospecific and non-MHC-restricted CTLs. However, antibody NK9 reacts with all leukocytes, at least at low intensity, and its specificity for cytotoxic cells appears to be more quantitative than qualitative (E. Säkselä and L. Lanier, personal communication).

The majority of NK cells express at low density the 46-kDa antigen CD38 recognized by antibody OKT10, whereas resting T cells are negative

for this antigen (89). However, the antigen is strongly expressed on both T and NK cells activated *in vitro* to proliferate (190, 191). Like T cells (190), *in vitro* activated and proliferating NK cells express HLA-DR, transferrin receptor, 4F2 antigen, and IL-2 receptor TAC (CD25) (78, 191, 192). CD25 antigen and transferrin receptor are rapidly down-modulated when the cells revert to a resting state (78), whereas HLA-DR antigens are maintained for a longer time (100), possibly due to a longer half-life of these molecules at the cell surface.

Although minor subsets of $CD3^+$ cells have been reported to mediate very low levels of non-MHC-restricted cytotoxicity, virtually all natural cytotoxicity is mediated by $CD3^-$ lymphocytes (95, 117, 183, 185). No anti-CD3 antibodies, regardless of their specificity, have ever been described to react with NK cells, although the transcript of the CD3ϵ gene, encoding one of the four chains of the CD3 antigen, has been reproducibly detected in $CD3^-$ NK clones (193) and in bulk cultures of NK cells (194).

Analysis of TCRβ and γ genes showed no evidence of rearrangement in both fresh and cultured human NK cells (195–200), although, as expected, rearrangement was observed in $CD3^+$ T cell clones with non-MHC-restricted cytotoxic activity (195, 200). A germ-line organization of the TCRα genes in at least most NK cells is suggested by the lack of significant decrease of hybridization of NK cell DNA to the TCRδ cDNA probe in Southern blotting, contrary to what would be expected if the δ genes were deleted following TCRα rearrangement (194). No rearrangements were evident in the TCRδ region of NK cells (194, 201). T cell clones that rearrange the TCRγ and δ genes and express TCR$\gamma\delta$ at the cell surface have been originated from either adult or fetal blood and shown to mediate non-MHC-restricted cytotoxicity (202–205). However, fresh TCR$\gamma\delta^+$ PBLs do not mediate natural cytotoxicity (L. Lanier, personal communication).

NK cells do not have detectable TCR on the cell surface, as detected by antibodies to TCR$\alpha\beta$ or $\gamma\delta$, nor do these cells express TCR after *in vitro* culture (100). Whereas no transcripts for the TCRα and γ genes are detectable in NK cells, a nonfunctional 1.0-kb transcript of the TCRβ gene, containing no V region, is reproducibly detected in NK cells or $CD3^-$ NK cell clones (100, 195, 196). Whether the truncated message in NK cells is derived from a partial D-J rearrangement in the TCRβ gene or is due to transcription of a germ-line gene is not yet known. Interestingly, the TCRβ gene in NK cells is more methylated than in T cells, but less methylated than in B cells and monocytes, suggesting at least a partial activation of the gene (206). NK cells also express large amounts of truncated TCRδ transcripts of different sizes, containing J and C regions, but no V region (194, 201). A possible difference between

T cells and NK cells in the 3′ untranslated region of TCRδ mRNA is suggested by the sequence data obtained by analyzing a limited number of transcripts cloned so far (194).

C. Surface Phenotype of NK Cells from Experimental Animals

Several antigens specifically expressed on mouse NK cells have been described. Alloantibodies different from anti-Lyt-2 and reacting specifically with NK cells were first described in anti-Ly-2 antisera generated by immunizing C3H mice with cells from the CE strain (207). A more specific antiserum (C3H × BALB/c) F_1 anti-CE, which lacks Ly-2 specificity, was prepared and used for the designation of a specific NK cell alloantigenic system, NK-1 (208). A monoclonal anti-NK-1 antibody, clone PK136, was also obtained (209). Sorting of spleen cells reactive or nonreactive with anti-NK-1 antibody from mouse strains expressing the NK-1.1 allele demonstrated that all the NK cytotoxic activity was present in the NK-1.1$^+$ subset (210). Anti-NK-1.1 antibody is specific for murine NK cells and reacts with a small population of spleen cells with granular lymphocyte morphology (210). Repeated weekly treatment *in vivo* of mice with anti-NK-1.1 antibody induces disappearance of mature NK cells, but not of NK cell precursors (211). NK cell-specific antibodies detected in CE anti-CBA alloantisera have been originally designated anti-NK-1.2 and considered to be specific for an allele of NK-1.1 (212). However, the existence of strains such as C57BL that react with both antibodies excluded the possibility that the two antigens were alleles (213). The strain specificity of the CE anti-CBA alloantiserum is similar, but not identical, to that of the NZB anti-BALB/c (anti-NK-2.1) sera (214). The two alloantisera are now considered to be directed against the same alloantigen, NK-2.1 (213). A third antigen, NK-3.1, which segregates independently of NK-1.1 and NK-2.1, was detected in several strains by a C3H anti-ST alloantiserum (215). When tested in the presence of complement against the appropriate strains of mice, these three antisera completely abolish the NK cell activity against YAC-1 and some other target cells. However, when other target cells, such as K562, were tested, the cytotoxic activity in C57BL spleens was eliminated by anti-NK-2.1 but not by anti-NK-1.1. Further, when RBL-5 and other target cells were used, neither antiserum affected NK cell activity. These results suggest a heterogeneity in the expression of the NK-1 and NK-2 antigens in murine NK cells (216).

The three antigens of the NK series are all alloantigens and therefore each is present only in a limited number of strains. Recently, a rat monoclonal antibody has been produced directed against an 87-kDa antigen, LGL-1, present on NK cells from all mouse strains tested (217),

although some strains express it at a higher density than others (V. Kumar, personal communication). The LGL-1 antigen is specifically expressed on most or all NK cells, although a small subset of $CD3^+$ $LGL\text{-}1^+$ spleen cells was detected by immunofluorescence (217).

Heterologous anti-asialo-GM_1 antisera together with complement completely eliminate murine NK cell activity and partially abrogate CTL activity (218, 219). Flow-cytometric studies have shown that asialo-GM_1 is expressed on both NK cells and CTLs, but that NK cells are more sensitive than CTLs to treatment with anti-asialo-GM_1 and complement (220). Asialo-GM_1 is also present on activated and tumoricidal macrophages (221). However, even with these limitations of specificity, the anti-asialo-GM_1 reagents were an extremely useful tool for dissecting the role of NK cells *in vivo* and *in vitro* before monoclonal antibodies to more specific antigens became available (219, 220, 222). Also, on the basis of asialo-GM_1 expression, a possible heterogeneity of murine NK cells was detected, i.e., whereas all NK cells cytotoxic for YAC-1 cells are asialo-GM_1 positive, those lysing herpes simplex virus (HSV)-1-infected fibroblasts include both asialo-GM_1-positive and -negative subsets (223). The reduced specificity of the anti-asialo-GM_1 antisera might rest in their reactivity with other gangliosides, such as asialo-GM_2 and asialo-GM_3 (224). Monoclonal antibodies have been produced that are more specific for asialo-GM_1 than the antisera and can completely deplete NK cell activity: These monoclonal antibodies have a lower reactivity to T cells than the polyclonal antisera and could be more specific reagents for murine and rat NK cells (224, 225).

Another antigen that is shared by NK cells and a subset of T cells, including CTL precursors and some MHC-restricted CTLs, is the Qa-5 antigen (226, 227). An IgM monoclonal antibody, anti-Qa-5, produced by AKR mice immunized with C57BL/6 lymphocytes is a useful reagent, together with complement, to eliminate NK cell activity (228). However, the pattern of expression of Qa-5 on NK cells from different mouse strains was found to differ from that observed on lymph node cells but to match that observed for the strain distribution of the NK-1.1 antigen, raising the possibility that the Qa-5 antigenic determinant is an epitope of the NK-1 molecule on NK cells (229).

Ly-11, a cell surface marker present on 10–20% of the cells from various lymphoid organs, is also expressed on NK cells and prothymocytes, but not on mature B or T cells (230).

Thy-1 antigen has a variable distribution on NK cells, with some but not all monoclonal anti-Thy-1 antibodies reacting with up to 50% of the NK cells in normal mice and up to 90% of NK cells in nude mice (231–233). The $Thy\text{-}1^+$ subset of NK cells has higher cytotoxic activity

and proliferative ability than the Thy-1$^-$ subset (234). IL-2-activated NK cells are mostly Thy-1$^+$, suggesting that either Thy-1 is an activation antigen on NK cells or that IL-2 induces preferential growth of Thy-1$^+$ NK cells (234).

Ly-5 antigen, a polymorphic determinant of the T200 molecule, is expressed on all hematopoietic cells, including NK cells. Anti-Ly-5 and nonpolymorphic anti-T200 antibodies block NK cell activity even in the absence of complement (208, 235). The T cell antigens Qa-2, Qa-4, Ly-6, and Ly-10, but not Qa-1, Qa-3, or Ly-2, are also expressed on a proportion of NK cells (232). Ly-1 (CD5) antigen, present on thymocytes, T cells, and a subset of T cells, is usually not present on NK cells (235), although its expression on 25% of NK cells has been reported in Ly-1.1 congenic mice (232). B cell/macrophage antigen Ly-M and the Lyb-2 B cell-specific marker are not detected on NK cells (232). The antibody MAC-1, specific for the C3bi receptor on myelomonocytic cells, detects this antigen on NK cells obtained from the peritoneal exudate of *Listeria* monocytogene-infected mice (236, 237). However, only a minor subset of fresh, non-activated NK cells was found to express the MAC-1 antigen (229).

Several cloned lines with NK cell characteristics have been generated from mouse lymphocytes (238–241). These cell lines have been shown to express both NK cell markers, such as NK-1.1 and NK-2.1, and T cell markers (239). Analysis of the rearrangement of TCR genes in these clones has definitively confirmed that most or all of these cell lines present functional rearrangements in the TCR genes, demonstrating that they are T cell clones with non-MHC-restricted cytotoxic ability (242, 243). The isolation of these T cell clones expressing NK cell markers may be due to the selective growth of the small subset of T cells expressing NK-1 and NK-2 antigens. The expression of these antigens is similar to that of the human NKH-1/Leu-19 antigen, which is usually found to be associated with T cell clones with non-MHC-restricted cytotoxicity. All studies performed on freshly isolated murine NK cells or on their short-term bulk cultures have confirmed that in mice, as in humans, NK cells neither functionally express nor rearrange any of the four TCR genes (234, 244, 245). Murine NK cells accumulate a nonfunctional 1.0-kb truncated TCRβ transcript but, unlike human NK cells, do not accumulate transcripts of any of the CD3 genes at detectable levels (244).

Murine NK cells, like human NK cells, can mediate ADCC, suggesting that at least a proportion of them bear FcγR (115, 246–248). The presence of FcγR on murine NK cells has also been suggested by data showing that absorption on monolayers of IgG-sensitized erythrocytes significantly reduces, but never completely abolishes, the NK cell activity of murine

spleen cells (246) and that a relevant proportion of the murine splenic lymphocytes that form conjugates with YAC-1 belong to the subset that forms rosettes with IgG-sensitized erythrocytes (249). Recently, it has been shown that fresh and cultured murine NK cells react with the anti-FcγRII antibody 2.4G2 (250). Northern blot analysis has shown that *in vitro* propagated murine NK cells accumulate transcripts only for FcγRII-α, one of the two genes encoding the 2.4G2-reactive FcγRII in the mouse (130). Thus, murine NK cells, like human NK cells, express only one type of FcγR. FcγRII-α is highly homologous to the CD16 FcR expressed on human NK cells (124). FcγRII-α-encoded polypeptide is the receptor used by murine NK cells in ADCC, as shown by the ability of the 2.4G2 antibody to block the ADCC activity of murine NK cells (130).

Another type of spontaneously occurring cytotoxic cells has been described in the mouse and termed natural cytotoxic (NC) cells. NC cells are not active against NK-sensitive target cells such as YAC-1, but preferentially lyse another set of target cells, of which WEHI-164 is the prototype (251, 252). NC cells are present in animals lacking NK cells, such as neonatal or beige mice, and were reported originally not to express markers typically expressed on NK cells, such as NK-1, NK-2, Qa-5, or Thy-1 (252–255). However, these early studies were performed using negative selection with complement and were therefore unable to distinguish the contribution of distinct subsets of NC cells. Positive and negative selection experiments by fluorescence-activated cell sorter have shown that NC activity can be mediated by a variety of phenotypically distinct NC cell subsets, including Thy-1$^+$ and Qa-5$^+$ cells (256). WEHI-164 is a very sensitive target for tumor necrosis factor (TNF), and the cytotoxic activity of NC cells has been shown to be mediated by TNF (257, 258). Thus, NC cells are a heterogenous group of cells able to produce TNF and therefore may include macrophages; T, B, and NK lymphocytes; and basophils (78, 258–260).

In the rat, NK cells have a surface phenotype similar to that of human and murine NK cells. Rat NK cells are asialo-GM_1^+ and do not express the T cell-associated antigens CD4, CD5, and CD25 (IL-2 receptors) or class II MHC antigens (261, 262). However, all rat NK cells express CD8 (OX8) antigen, unlike murine NK cells, which are CD8 (Ly-2) negative, or human NK cells, which express CD8 at low density in only 30–50% of the cells. Rat NK cells, like human and murine NK cells, do not express or rearrange the genes encoding the TCR (263, 264). In the dog NK cells express some markers of T cells, including CD8 (265). Cells with NK cell activity and LGL morphology which are distinct from classical T cells have also been demonstrated in the horse, miniature

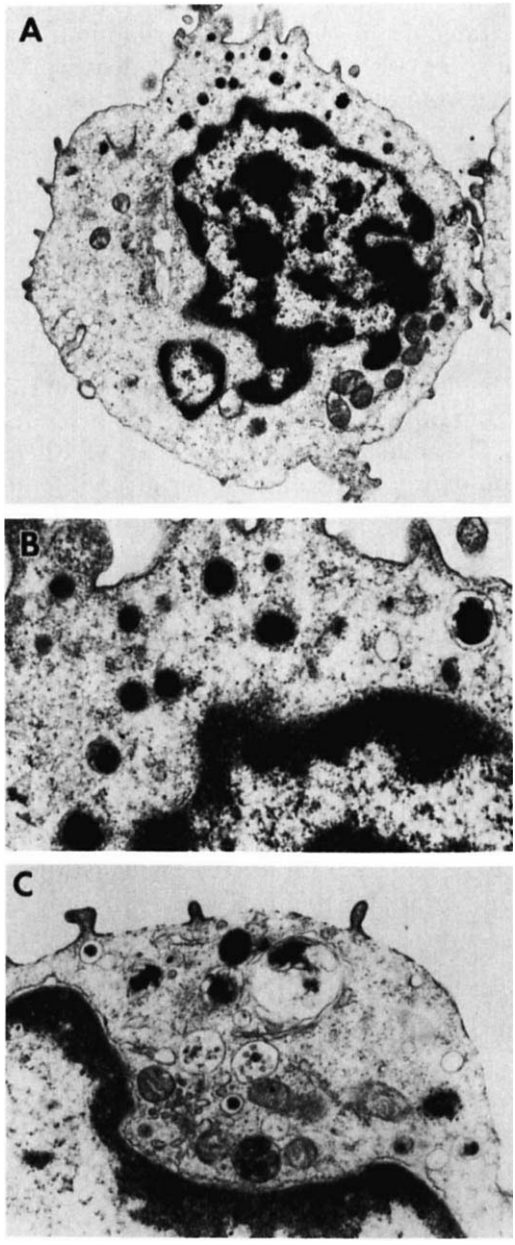
swine, and other mammalian species (266–268). Cytotoxic cells possibly corresponding to NK cells have also been identified in birds (269), amphibians (270), and fish (271).

D. Morphology and Cytochemistry of NK Cells

The morphological identification of most NK cells as LGLs was originally determined by analysis of lymphocytes binding to NK cell-sensitive target cells (35, 83, 84). The central role of LGLs as NK effector cells was suggested also by the positive correlation between the number of LGLs able to bind to K562 target cells and the level of cytotoxic activity among normal donors (84), and by the finding that both NK cell activity and LGLs are recovered in the same fractions when human PBLs are separated by centrifugation on discontinuous density Percoll gradients (85). At least 70% of the human peripheral blood LGLs have been shown to have NK cell activity (90). LGL morphology has also been shown in NK cells from experimental animals, including mice (272), rats (273), and horses (266).

Human peripheral blood cells with LGL morphology, i.e., with a high cytoplasm–nucleus ratio, indented nucleus, and azurophilic granules, were described in 1911 by Pappenheim and Ferrata (274) and termed monocytoid or leukocytoid lymphocytes. In transmission electron microscopy human LGLs appear as medium-sized lymphocytes with round or indented nuclei, condensed chromatin, and unusually prominent nucleoli (275–278) (Fig. 5). The cytoplasm is abundant and contains a variety of organelles. A well-developed Golgi apparatus with many smooth and coated vesicles is usually found in the nuclear notch. Prominent centrioles and associated microtubules are also detected in this area. The cytoplasm contains abundant mitochondria and a number of lysosomal organelles. Common among these are membrane-bound granules containing a homogeneous electron-dense matrix, but other structures, such as smooth vesicles, coated vesicles, and multivesicular bodies, are also normally present (275–278). The granules present typical internum and externum. In resting mature NK cells granules range in size from 50 to 800 nm in diameter, display a circular to elongated profile,

FIG. 5. Ultrastructural features of NK cells from a short-term (10-day) bulk culture of human PBLs. (A) ×9,100. (B) Details of the granules; ×26,000. (C) Details of the granules of NK cells treated for 20 minutes with anti-CD16 antibodies coupled with Sepharose. Most granules have lost their electron-dense core and present membrane formations, myelin figures, and multivesicular bodies, characteristic structures in activated and, sometimes, resting NK cells; ×26,000. (Electron-microscopic preparations were by C. Grossi and B. Perussia, modified from Ref. 100 with permission from Karger, Basel, Switzerland.)


A
B
C

and contain an electron-dense core (internum) surrounded by a layer of less opacity (externum) (275, 276, 279, 280). The matrix of the granules is usually separated from the limiting trilaminar membrane by an electron-lucent space, within which electron-dense spikes can sometimes be seen radiating from the matrix to the membrane. The electron-dense core matrix is usually amorphous, except in cases in which a paracrystalline structure can be identified (279, 280). Various organelles which are probably a modification of the same granules are, however, observed in NK cells and have been attributed either to differentiation stages of the granules, similar to observations in granules of basophils, or to activation of the NK cells during lymphokine treatment or cytotoxic activity (275, 279, 281). Vesicles are observed, usually still containing a residual electron-dense matrix surrounded by small vesicles, to form multivesicular bodies, membrane myelin figures, and tubular structures. NK cells also contain numerous electron-lucent pinocytic vesicles and large vacuoles. The contents of the vacuoles are very heterogeneous, containing electron-dense particulate material and cellular debris. The endocytic nature of these structures was confirmed by the demonstration of Percoll beads in the vacuoles of LGLs incubated for 1-2 hours at 37°C in the presence of high concentrations of Percoll (276). Although NK cells and LGLs were originally described as nonphagocytic, being unable to phagocytize latex beads, opsonized RBCs, or immune complexes (275, 278, 282), several studies have demonstrated at least a limited ability of these cells to phagocytize 2-aminoethylisothiouronium bromide hydrobromide (AET)-treated SRBCs, opsonized *Staphylococcus aureus*, and complement-coated bacteria (277, 280, 283, 284).

Typical structures described in LGLs are the parallel tubular arrays (PTAs). These structures, originally described in PBLs (285, 286), were demonstrated to be a marker for FcγR-bearing lymphocytes (277, 278). The PTAs are quite variable in overall size, with some as large as 1.3 μm in diameter or 1.7 μm in length. Lymphocytes contain large PTAs, small PTAs, or both. All of the PTAs contain a tubular substructure formed by tubules packed in wall-to-wall contact and usually located in the notch region of the nucleus in close association with the centriole and the Golgi apparatus (276-278, 280, 287-290). Some of the PTAs are surrounded by a membrane, but, most often, distinct membranes are difficult to identify. Inclusions containing only tubules were termed type A PTAs, whereas other inclusions containing tubules and homogeneous electron-dense material were termed type B PTAs (287). Comparison of the diameters of the tubules in different reports reveals extreme heterogeneity, with measurements ranging from 13 to 44 nm. However, most papers report a diameter of either ~16 nm (276, 277, 287, 289) or ~40 nm

(280, 288, 290), suggesting the possibility of two classes of PTAs. Isolated reports of PTAs in only a very limited number of LGLs or in the complete absence of these structures (275, 279) have been attributed to the failure to use either ultrathin sections and high magnification or ammonium chloride for lysing RBCs in the cell preparation, which induces disappearance of the PTAs for a certain time after treatment (289, 291). However, the heterogeneity of the PTAs and the different proportions of LGLs containing PTAs reported by various authors may suggest that the PTA structure is unstable and may be affected by many different factors involved in the preparation of cells.

Recently Caulfield *et al.* (279) and Kang *et al.* (280) reported the presence of crystalline lattice or gratings in a proportion of granules from NK clones and peripheral blood LGLs, respectively. In stained sections crystalline structures were seen in about 10% of the densest granules, but in unstained sections all of the densest granules contained these structures (279). The lattices were composed of hexagonally packed points, each point equidistant from six other points. The gratings consisted of a set of parallel lines, usually straight, but occasionally in a whorled fingerprint pattern. The grating pattern also appeared to be superimposed on the lattice pattern, with the sets of parallel lines running in three directions that were at 120° with respect to one another. Lattice and gratings have been demonstrated to be simply different views of the same structures (279). The lattice spacings average 6.9 ± 0.3 nm, with a thickness of the electron-dense lines or points ranging from 2.5 to 3.6 nm. In addition to hexagonally packed lattices, cubic lattices were seen occasionally (279). In some granules the lattice appears smaller, with a looser packing, a microtubular appearance, and a diameter of 17 nm, similar to the PTA microtubules described by other authors. Frequently, the gratings or lattice unraveled and formed tubules and trilaminar strands of unit membrane that extended from the core of the granules (279). These laminar membranes, myelin figures, vesicles, and multivesicular bodies, in the presence or absence of residual crystalline dense material, are more frequently seen in activated NK cells (281) or in discharged granules during cytotoxic activity (279). These results strongly suggest that granules with PTAs, membranes, or lattices in NK cells may be in fact structural variants of a single granule type (279). The crystalline lattices most probably are formed by the phospholipids that give rise to the membranes and vesicles. Phospholipids in model lipid–water systems form either lamellar or nonbilayer arrangements (292). The phospholipids in nonbilayer arrangements form long rods, with the head groups and water in the center and acyl chains radiating outward, and the rods are then ordered in hexagonal or cubic

arrays (Hex II phases), with center-to-center spacing of 4.5-7.0 nm (292). Alternatively, the rods may have the head groups on the outside (Hex I phase): In thin sections, this phase appears as an electron-dense honeycomb (279, 292), with a center-to-center distance of 16-17 nm, similar to the structure observed in some cases of PTA.

Because antibody HNK-1/Leu-7 has been shown to react with most of the PBLs with LGL morphology, although only a part of them represents NK cells, several studies have analyzed the morphology of the two subsets of Leu-7$^+$ cells: the Leu-7$^+$, CD3$^+$ T cells and the Leu-7$^+$ CD16$^+$ NK cells (280, 293-299). In most studies Leu-7$^+$, CD16$^+$ NK cells were shown to have the typical LGL morphology with numerous electron-dense granules, PTAs, and phagocytic ability for opsonized bacteria, whereas Leu-7$^+$, CD3$^+$ T cells have fewer granules than NK cells, no PTAs, and no phagocytic ability (280, 294-299). One study (293), however, reports that Leu-7$^+$, CD3$^+$ T cells have LGL morphology with PTAs, whereas most Leu-7$^+$, CD16$^+$ or CD11b$^+$ NK cells present a low number of granules and no PTAs.

The granules of NK cells stain for glycoproteins, acid phosphatase, trimetaphosphatase, arylsulfatase, and β-glucuronidase, indicating that they are primary lysosomes (275, 276, 280, 298, 300). The presence of endogenous peroxidase in LGLs has been reported in some (276, 280, 284) but not in many other (84, 275, 278, 301) studies. Babcock and Phillips (276) could detect peroxidase activity in only a few vacuoles and suggested that it could represent enzymatic activity of phagocytosed material and not endogenous peroxidase.

NK cells express various esterase activity, as detected by cytochemistry. Naphthol AS-D chloroacetate esterase, an enzyme characteristic of neutrophilic granulocytes, is also expressed at least in some granules of NK cells (87, 276). Nonspecific esterases, both α-naphthyl acetate (ANAE) and α-naphthyl butyrate (ANBE), are detectable in NK cells, although sometimes discordant results were obtained, depending on whether optical or electron microscopy was used in the analysis (84, 275, 276, 302). ANAE and ANBE are present in the granules of LGLs, giving a scattered staining often ignored in optical microscopy, and, as an ectoenzyme, in the membrane. Like monocyte esterase (303), the membrane activity but not the granule-associated activity is inhibitable by sodium fluoride (NaF) (275, 276). The staining pattern of LGLs is very different from that observed on most T cells, in which the esterase activity appears as a single discrete and NaF-noninhibitable cytoplasmic dot, corresponding ultrastructurally to the Gall body (a cluster of lysosomes adjacent to a lipid droplet) or to clustered dense bodies (303, 304).

IV. Origin and Differentiation of NK Cells

A. Tissue Distribution

Morphological evaluation of the peripheral blood of normal donors originally indicated that LGLs represent ~3.6% of the lymphocytes (90), and a similar proportion was detected when the number of cytotoxic NK cells was evaluated by single-cell cytotoxic assay (60). However, the use of monoclonal antibodies, such as anti-CD16 and NKH-1, has shown that these cells represent a much larger proportion of total PBLs. $CD16^+$ lymphocytes represent, on average, 15% of PBLs, with large variability among donors (ranging from 2 to 50%) (95, 114, 117, 118). The large majority of $CD16^+$ PBLs have LGL morphology and lack B or T cell markers (95, 114). More than 60% of $CD16^+$ PBLs freshly separated and more than 80% of IFN-treated $CD16^+$ PBLs bind to K562 cells (95) and the majority of these cells are cytotoxic in a single-cell assay in agarose (305). The spleen is one of the major sources of NK cells in both humans and experimental animals. $CD16^+$ cells in human spleen represent 3–4% of the total lymphocytes (95). LGLs localize in the spleen red pulp and not in the major areas of T cell recirculation, i.e., spleen white pulp and lymph nodes (95, 306, 307). $CD16^+$ cells and NK cell activity are absent from the lymph nodes of healthy individuals or animals (95, 308). NK cells are not present in recirculating thoracic duct lymphocytes, but treatment with IFN *in vivo* may induce the appearance of a small number of NK cells in the thoracic duct (309). In bone marrow, the number of $CD16^+$ lymphoid cells is very low, ~1% of the mononuclear cells, and the cytotoxic activity is also very low (95, 308, 310). $CD2^+$ lymphocytes sorted from bone marrow have an enriched NK activity, whereas $Fc\gamma R^+$ cells are inactive, perhaps suggesting the presence of immature $CD16^+$ NK cells in the bone marrow (310). The low activity of NK cells in the bone marrow might also rest in the presence of NK cell-sensitive target cells in the bone marrow that determine a functional inactivation of the NK cells (76). In the mouse, $NK1.1^+$ bone marrow cells can be purified by fluorescence cell sorting and shown to have cytotoxic activity comparable to that of spleen $NK1.1^+$ cells (229).

In the human tonsilla palatina, the number of $CD16^+$ lymphocytes and NK cell activity is reduced (95, 311, 312). Leu-7^+ cells are present in the tonsils, some with small lymphocyte and some with LGL morphology (311). The Leu-7^+ cells with LGL morphology are found in the crypt epithelium, whereas the small Leu-7^+ are located in the germinal

center (311). The contribution of Leu-7$^+$ LGLs to cytotoxicity from tonsil cells is, however, not clear, and macrophages might account for some of the observed cytotoxicity (312). Germinal center Leu-7$^+$ cells, in tonsils and lymph nodes, belong to the CD3$^+$, CD4$^+$, Leu-7$^+$ subset of noncytotoxic T cells (154).

NK cell activity has been detected, at least in long-term assays, in both the airspace and the interstitial compartments of the lung (313). However, analysis using anti-CD16 monoclonal antibodies suggests that typical LGLs or NK cells are present primarily in the lung interstitium (314). The NK cell population in the lung is responsive to locally derived regulatory factors (e.g., intratracheal virus infection or IL-2 administration), but relatively unresponsive when systematic routes of administration of lymphokines or viruses are used (315).

LGLs and NK cells have been demonstrated in the intestinal mucosa of mice (316) and rats (317). In the rat large intestine, LGLs represent up to 25% of the intraepithelial lymphocytes (318). The murine LGLs from intestinal epithelium have the same surface phenotype as spleen NK cells (319). Although the number of mucosal NK cells is low, their participation in the local defense against murine enteric coronavirus has been demonstrated (320). Peritoneal exudate cells are also a good source of NK cells. However, several studies of humans have failed to show elevated NK cell activity or CD16$^+$ cells in intestinal mucosa lymphocytes (321–326). Shanahan *et al.* (326) demonstrated that human mucosa NK cells are NKH-1/Leu-19$^+$ but CD16$^-$, and that CD3$^-$ non-MHC-restricted cytotoxic cells can be generated from CD2$^+$, CD8$^+$, CD16$^-$, NKH-1$^-$ cells, possibly representing pre-NK cells.

Nonparenchymal cells from murine and rat liver kill both the NK cell-sensitive target cell YAC-1 and the NK cell-resistant P815 cell (327–332). The killing of both target cells was attributed to asialo-GM_1-positive cells with characteristics (e.g., density, half-life, kinetics of killing, age dependence, and nonadherence) typical of NK cells (327, 330). The pattern of target cell specificity of these liver NK cells suggests that they, unlike peripheral blood and spleen NK cells, might be in an activated state (328, 331). Macrophages (Kupffer cells) were usually cytotoxic only after activation *in vivo* by stimulants such as *Corynebacterium parvum* (328, 329, 332). In the rat, the LGLs or NK cells in the liver have been found to be identical to the previously described liver "Pit cells" (333–335). These cells are CD8$^+$, CD5$^-$, mostly asialo-GM_1-positive cells with the morphology of LGLs and typical electron-dense acid phosphatase-positive granules (335). Pit cells are contained in the liver sinusoid, where they are in close interaction with endothelial cells and present fingerlike extensions that penetrate through the sinusoid

endothelial cells (334). In liver cell suspensions obtained by enzymatic dissociation, LGLs represent 5.3% of the cells, but their proportion in cell preparations obtained by high-pressure liver perfusion is up to 30% (334).

Treatment of mice with biological response modifiers such as maleic anhydride divinyl ether (MVE-2) or *C. parvum* induces a dramatic augmentation of liver NK cell activity 3–5 days after treatment (327). The increase in NK cell cytotoxicity corresponded to a ten- to 50-fold increase in the number of lymphoid cells with LGL characteristics that were isolated from enzymatically digested suspensions of perfused liver (327). The phenotype of the isolated LGLs is the one typical of NK cells, i.e., asialo-GM_1^+, Thy-1^+, Ly-5^+, Qa-5^+, MAC-1^+, Ly-1^-, Ly-2^-, L3T4^- (327).

The migratory pattern of NK cells has been studied by adoptive cell transfer studies, using purified radiolabeled rat LGLs from blood or spleen (306, 313). Following intravenous injection, more LGLs than T cells localized in the capillary bed of the lung, but fewer LGLs migrated to the spleen, where, unlike T cells, they localized in the red pulp (306). The adoptively transferred LGLs did not appear in the thoracic lymph. While NK cells do not appear to recirculate, levels of NK cell activity can be dramatically altered in various organ sites following administration of immunostimulants (327, 336). The mechanisms responsible for these alterations could be increased activity or proliferation of preexisting NK cells, localization of blood-borne NK cells or NK cell precursors, or redistribution of mature NK cells from one site to another. In the case of increased NK cell activity and numbers in the liver following MVE-2 or *C. parvum* treatment, it was shown that the accumulation of LGLs in the liver is not affected by splenectomy, but is prevented by ^{89}Sr-induced destruction of the bone marrow environment, suggesting that the accumulation is due to migration in the liver of NK cells recently derived from bone marrow progenitors (336).

The pattern of migration of NK cells suggests specific interaction with endothelial cells. Some T cells interact with high endothelial venules in the lymphoid tissue by expressing a homing receptor recognized by antibody MEL 14 (235). NK cells from normal mice do not express MEL 14 antigen, but up to 10% of IL-2-propagated NK cells from *scid* (severe combined immunodeficiency) mice express it (229). NK cells also express LFA-1 antigen, which has as a putative ligand intercellular adhesion molecule (ICAM-1) found on endothelial cells (178). The epitope recognized by HNK-1/Leu-7 antibody has also been involved in various systems of intercellular interaction and might play some role in LGL migration (169).

B. Age, Gender, and Genetic Control of NK Cells

Information on human NK cells during fetal development is very fragmentary. Marginal cytotoxic activity against K562 cells was observed in fetal liver cells at 8–11 weeks of gestational age, and higher cytotoxicity was observed with liver cells from an 18-week fetus, especially after stimulation in mixed-leukocyte culture (337). At no time was cytotoxic activity mediated by fetal thymus cells (337). In peripheral blood, no activity was observed in 20-week fetuses, even after boosting with IFN-γ (338). However, induction of cytotoxic cells was observed with IL-2 treatment (338). In premature infants at 27 weeks of gestation, NK cell activity was constitutive in peripheral blood and was augmented by IFN-γ or IL-2 treatment (338). At birth, cord blood lymphocytes usually have normal ADCC activity, but NK cell activity against K562 target cells ranges from severely depressed to normal (95, 339–341). However, cytotoxic activity mediated by cord blood lymphocytes against another NK cell-sensitive target cell line, MOLT-4, was found to be comparable to that of adult PBLs (340). In cord blood, about 19% of the lymphocytes are CD16$^+$, similar to the proportion observed in adult PBLs (95). However, the HNK-1/Leu-7 antigen was not expressed on CD16$^+$ cord blood lymphocytes (95, 342). The number of LGLs in cord blood is also comparable to that of adult peripheral blood (341); therefore, NK cells are present in normal number in cord blood and their low cytotoxic efficiency may depend on immaturity (339) or the presence of suppressor cells (341). In the miniature swine, as in humans, at birth lymphocytes mediate ADCC but not NK cell cytotoxicity (267). If the piglets are hysterectomy derived and maintained germ free, NK cell activity appears only after 4 weeks of age, whereas piglets maintained in the standard specific pathogen-free animal colony develop NK cell activity at 2–3 weeks, suggesting that stimulation by the microbial flora and environment plays some role in the maturation of NK cells (343). In humans the proportion of CD16$^+$ cells in PBLs remains relatively constant after birth, whereas the number of Leu-7$^+$ cells increases almost linearly with age (148). A modest increase in NK cell cytotoxicity was observed in individuals more than 80 years old (344–349). The Leu-7$^-$ CD16$^+$ subset, with the highest cytotoxic activity was not increased in these subjects, whereas a significant increase was observed in the Leu-7$^+$ CD16$^+$ NK cell and Leu-7$^+$ CD16$^-$ T cell subsets (345–347, 349, 350). This increase in NK cell activity in older individuals may reflect a normal maturation of the NK cell system, or a preferential survival of subjects with elevated NK cell activity. In the age group between 20 and 60 years,

male donors have a higher proportion of both $CD16^+$ lymphocytes and NK cell activity than do female donors (56, 95, 344, 345, 351).

Although the relative cytotoxic ability of healthy donors is relatively constant when tested at different times (52), circadian and circannual rhythms of NK cell activity have been demonstrated for both human and murine NK cells (352-355). The maximum of activity for human donors occurs early in the morning and with a second minor peak in the afternoon, with a peak-to-trough difference of 50% or more of the average activity (352). These data point to the importance of collecting control donors and patients' blood at similar times during the day when sequential studies are performed.

Accurate genetic studies are lacking in humans, but a partial correlation between NK cell activity and the presence of certain HLA alloantigens has been reported (56, 356-358).

In mice spleen cells at birth are devoid of NK cell activity, and this activity cannot be detected during the first 11 days of life (359, 360). A small fraction of mice develop marginal splenic NK cell activity between 12 and 21 days, and all mice show NK cell activity at 26-28 days, although lower than observed at its peak at 6-10 weeks of age and afterward continuously declining with age in most strains of mice (359-361). However, SM/J (362) and AKR (363) strains do not show this decline, and the decrease is less rapid in the peripheral blood of all strains (364). IL-2 and IFN can induce NK cytotoxic cells in cultures of spleen cells from old mice, suggesting that asialo-GM_1-positive pre-NK cells are present in the spleen (365-367). The failure of NK cells in old mice to mediate cytotoxicity has been variably attributed to the presence of suppressor cells (367, 368), a loss of competence to lyse target cells (369, 370), or a change in the regulatory interaction between NK cells and other cell types (371).

Mouse NK cell activity is under polygenic control, with at least two controlling genes associatad with the D locus of the H-2 region, and high responsiveness is usually dominant over low responsiveness (372-375). Analysis of the participation of non-H-2-linked genes in determining NK cell activity in $H\text{-}2^s$ congenic mice (376) revealed not only the dominance of the high NK cell activity phenotype over the low-activity phenotype in F_1 hybrid offspring, but also that crosses between two low NK cell strains were complementary in generating F_1 offspring with high or intermediate NK cell activity. Such genetic complementation between the low-activity NK cell pairs indicates that the low-activity NK cell phenotype in the various strains have different genetic bases. In the

SJL strain, three different non-MHC-linked genes were found to account for the poor responsiveness of NK cells to IFN (376).

C. Congenital Defects of NK Cells

Complete absence of NK cells is rare and has been described in only a few patients. Two of these patients, one male and one female, belong to a group of four siblings with recurrent viral infections (375). All four siblings developed infectious mononucleosis (IM) as young adults. A boy who had respiratory infections and progressive bronchiectasis since the age of 7 died at the age of 16 of complications of IM. All immunological parameters tested were normal, but NK cell activity was not tested. A sister and a brother also had recurrent viral infections and pneumonia and the sister had progressive bronchiectasis starting at age 7–10, before any evidence of Epstein-Barr virus (EBV) infection, as shown by the absence of anti-EBV antibody before developing IM. Both patients lacked NK cell activity against both K562 cells and HSV-1-infected target cells (375). No NK cells could be detected in peripheral blood of the sister 4 and 9 years after IM, using various monoclonal antibodies, including anti-NKH 1/Leu-19 and anti-CD16. All other immunological parameters tested were normal. A fourth brother developed IM at 21 years of age, but he was otherwise healthy. He had a somewhat reduced level of NK cell activity, which was augmented by *in vitro* treatment with IFN (375). Both parents were healthy and had normal NK cell activity (375). Biron *et al.* (377) have described another young female with severe viral infections, including varicella and cytomegalovirus, and complete absence of both NK cell activity and NKH-1/Leu-19$^+$ and CD16$^+$ lymphocytes. In all of these patients CD16 and CD11b antigens were normally expressed on neutrophils and on monocyte/macrophages.

NK cell activity is also deficient in patients with defective expression of the CD11/CD18 group of surface receptors (378); however, in these patients the clinical pathology is dominated by the defect in phagocytic cells and severe bacterial infections, and it is difficult to evaluate the role of the NK cell defect in the pathogenesis of the disease.

Depressed, but not absent, NK cell activity has been observed in patients with X chromosome-linked lymphoproliferative disorder (X-LPD) (379, 380). However, NK cell activity is normal in males at risk (i.e., sons of X-LPD heterozygous mothers with 50% risk of developing X-LPD after EBV infection). Thus, the defect in NK cell activity, apparently due to a lack of recycling ability rather than to a decrease in NK cell number, is acquired after EBV infection (379).

A specific NK cell hyporesponsiveness is observed in patients with Chediak-Higashi syndrome (CHS), a rare autosomal recessive disease

associated with cellular dysfunction, including fusion of cytoplasmic granules and defective degranulation of neutrophil lysosomes. Granules of the neutrophils are abnormally large, and clinical manifestations of the disease include defective pigmentation and increased susceptibility to infection (381). Humoral immunity and delayed-type hypersensitivity are normal, but children usually die of pyogenic infection, presumably resulting from their neutrophil abnormality. Survivors generally succumb to an LPD that may be malignant (382). NK cell activity in CHS patients is ten to 100 times lower than in normal controls (383-386): The number of NK cells is normal, as judged by the number of target binding cells and of cells positive with anti-NK monoclonal antibodies, but the number of cytotoxic cells is decreased, and the kinetics of lysis is slower than in normal NK cells (387-389). IFN increases the activity of CHS patients' NK cells (383, 387, 388). The NK cells in CHS patients characteristically contain a single, large granule in the cytoplasm (390): The decreased cytotoxic activity of these cells is probably due to a defect of the ability to secrete factors involved in cytotoxicity. Other lymphocyte-mediated functions in CHS patients appear to be normal, but the lysosomal defects can also be observed in granular Leu-7^+ T cells and in activated B cells (391).

In mice the functional activity of NK cells has been found to be modulated by several point mutations associated with coat color. The most commonly studied gene is *Bg*, which determines beige coat color (392). Mice carrying the *Bg* gene have been regarded as animal models for CHS. Homozygosity at the *Bg* gene determines defects in lysosomal membrane functions, resulting in granulocytes with giant lysosomes and abnormal functions and in altered melanosomal functions, leading to beige coat color (392). Beige mice have strongly depressed NK cell activity; the NK cell defect is post-target cell recognition and is only partially reversed by IFN (393, 394). Other B and T lymphocyte functions are almost normal. Beige mice have also been widely used as experimental models to analyze the role of NK cells *in vivo*. Of other color mutations in the mouse, leaden, fuzzy, and pale ears have no effect on NK cell activity, whereas satin (*Sa*) is also suppressive (395). When *Sa* and *Bg* are present in the same animal, their suppressive effect on NK cells is synergistic, but allospecific CTLs are also affected (395).

In various types of congenital B cell immunodeficiency (X chromosome-linked agammaglobulinemia, transient hypogammaglobulinemia, and most cases of ataxia telangiectasia and common varied immunodeficiency), NK cell activity is normal (396-405). Normal NK cell activity is also observed in patients with DiGeorge's syndrome, showing that functional thymus is not required for NK cell differentiation (398, 401, 406). The thymic independence of NK cells is also supported by the fact that

athymic nude mice and rats have stronger NK cell activity than do their euthymic littermates (360, 407). About half of the patients with Wiskott-Aldrich syndrome have normal NK cell activity against K562 target cells, but all of these patients have severely depressed NK cell activity against virus-infected target cells, despite normal IFN-α titers (405). Some patients with SCID show a depressed NK cell activity and some display normal or augmented activity (95, 399, 404, 405, 408-411). In SCID patients with elevated NK cell activity, most circulating lymphocytes have the characteristics of Cl1b$^+$ LGLs (409-411). These LGLs are probably in an activated state, as suggested by the presence of activation antigens and the resistance of their cytotoxic ability to functional inactivation by *in vivo* irradiation (409-411). Some of the interesting findings in SCID patients are the dissociation among NK cell activity against K562 cells, cytotoxicity against virus-infected cells, and ability to produce IFN (405).

In *scid* mice that lack both T and B cells, NK cell activity is normal (412, 413) and NK-2.1$^+$ NK cells comprise the large majority of spleen lymphocytes (229). Unlike the thymus of normal mice, that of *scid* mice contains cytotoxic NK cells that express typical NK cell markers, at least after short culture in the presence of IL-2 (229).

Microphthalmic (*mi/mi*) mice are congenitally osteopetrotic, with reduced marrow and a deficiency in natural killing (414). Experimentally, osteopetrosis and loss of NK cell activity can be observed by treating mice with 17β-estradiol for 6 weeks (414, 415). Estradiol-treated mice possess nonlytic, IFN-nonresponsive immature cells which express the NK cell-specific antigen NK-1.1, presumably arrested prior to a bone marrow-dependent stage of NK cell differentiation (210, 415).

D. Malignant Expansion of NK Cells

Acute leukemia with an NK cell phenotype has been described in only a few cases. Komiyama *et al.* (416, 417) described three cases in children with an acute course. The patients presented with lymphoadenopathy, splenomegaly, hepatomegaly, and 300,000-400,000 lymphocytes per cubic millimeter of peripheral blood. The circulating cells were CD3$^-$, CD4$^-$, CD8$^-$, CD16$^+$, CD11b$^+$, HNK-1/Leu-7 and mediated strong cytotoxicity against K562 target cells (416, 417). The cell morphology was lymphoblastic, without evident granules. Two continuous cell lines were generated from one of the patients, and these maintained cytotoxic activity and cell surface markers of NK cells. Another case of aggressive NK cell leukemia was described in an adult. The cells of this patient bore the typical LGL morphology and were CD3$^-$, CD4$^-$, CD8$^-$, CD16$^+$, HNK-1/Leu-7$^-$, with strong cytotoxic activity against K562 target cells (418). An IL-2-dependent cell line derived from this patient

was maintained for several months. All of these cases of acute NK cell leukemia were clonal in origin, as shown by analysis of chromosomal aberrations (416-418).

Cells from about half of the patients with chronic T cell lymphocytosis have LGL morphology and some of the markers or functions of NK cells. LGL lymphocytosis is usually manifested by granulocytopenia or RBC aplasia, thrombocytopenia, hypo- or hypergammaglobulinemia, and a relative or absolute increase in cells displaying LGL morphology (103). Since the first description of LGL lymphocytosis 10 years ago (419, 420), the number of reported cases of this disease, initially considered very rare, has increased and a large number of patients have now been described. In most cases of LGL lymphocytosis the phenotype of LGLs is rather homogeneous and these cells express CD3 and CD8 antigens. CD2 is expressed in cells from most patients, but the CD5 antigen present on all normal T cells and some B cells is usually absent or expressed at very low density on the LGLs. All cases of LGL lymphocytosis that express the CD3 antigen have rearranged TCR genes and express either TCR$\alpha\beta$ or TCR$\gamma\delta$, indicating the T cell origin of these cells (421-428). The heterogeneity of the LGLs in the patients, the frequent spontaneous remission observed, as well as the chronic and relatively benign course of the disease have led to the hypothesis that LGL lymphocytosis is not a malignancy, but a reactive process (429, 430). Although reactive LGL lymphocytosis can probably occur, for example, in B cell chronic lymphatic leukemia or EBV infections, cells in all tested patients with $CD3^+$, $CD8^+$ LGL lymphocytosis have unique rearrangements of the TCR genes, demonstrating the monoclonality of the disease (421-429).

The $CD3^+$ LGLs usually mediate efficient ADCC but low spontaneous cytotoxicity, if any (103). Surface antigens preferentially expressed on NK cells, such as CD11b, HNK-1/Leu-7, and NKH-1/Leu-19, are expressed on the cells from some patients. Cells from almost all patients express FcγR, as shown by rosette formation with IgG-sensitized RBCs or binding of immune complexes, and the FcγR is usually functionally active in mediating ADCC. Relatively few studies have tested anti-CD16 FcR antibodies, but among these, some (431, 432) reported reactivity of cells from most patients with anti-CD16 antibodies such as B73.1 or Leu-11, whereas in another study (433) cells from only a few patients reacted with antibody B73.1; instead, a more consistent reactivity was observed with AB8.28, an antibody reacting with a surface molecule different from CD16 antigen, but perhaps functionally related to the FcγR on NK cells and neutrophils (120). In another study, anti-Leu-11 antibodies were negative, but consistent reactivity was observed with the anti-CD16 CLB FcR-gran 1 antibody (434). The same phenotype

(CLB FcR-gran 1^+, Leu-11^-) was previously reported for $CD3^+$ clones with ADCC activity derived from healthy individuals (136). Unlike other anti-CD16 antibodies, the CLB FcR-gran 1 antibody reacts at low intensity with a proportion of T cells in peripheral blood, in addition to NK cells (G. Trinchieri, unpublished observations). Different types of FcγR (e.g., CD16 and CDw32) are highly homologous in their extracellular portions (124). In the mouse, antibody 2.4G2 reacts with both the product of FcRII-α, expressed on NK cells and macrophages, and the product of FcRII-β, expressed on B and T cells and macrophages (130, 250). It is possible that some of the anti-CD16 antibodies have similar specificity in humans. Thus, although $CD3^+$ cells from some patients probably express CD16 FcγR, in other patients a different type of FcγR might be used in mediating ADCC. The $CD3^+$ $CD16^+$ phenotype expressed by some patients might be an aberrant antigenic expression (lineage infidelity) by the malignant cells, as observed in many cases of leukemias from various cell lineages. Alternatively, monoclonal LGLs could originate from the T cell subset characterized by high CD3 and low CD16 antigen expression, and constituting a measurable subset in about 4% of healthy donors (135). The spontaneous cytotoxicity of $CD3^+$ LGLs is usually low and can be augmented by treatment with IL-2 or anti-CD3 antibodies (432, 434). The rare cases of LGL lymphocytosis with TCR$\gamma\delta$ have spontaneous cytotoxic activity that is blocked by anti-CD3 antibodies, analogous to observations of TCR$\gamma\delta^+$ clones with non-MHC-restricted cytotoxic activity (203, 205). Thus the TCR$\gamma\delta^+$ cells, unlike TCR$\alpha\beta^+$ cells, might use their TCR for non-MHC-restricted cytotoxicity in this form of LGL lymphocytosis (434).

Only about 10% of the patients with LGL lymphocytosis have cells with an NK cell phenotype ($CD3^-$, $CD16^+$, $CD2^+$, $CD8^+$ or $CD8^-$) which usually have high spontaneous cytotoxic activity and show no TCR gene rearrangements (423, 431, 432, 435, 436). However, evidence for monoclonality is given in several cases by chromosomal aberrations (435, 436). LGL lymphocytosis with an NK cell phenotype has been described in some cases to have a more benign course than does $CD3^+$ lymphocytosis, but other cases present the same hematopoietic, immunological and rheumatoid disorders present in the $CD3^+$ cases (436–438).

LGL lymphocytosis in some cases presents mostly in the form of T lymphomas (439–441). However, the organ localization of these lymphomas is not that typical of T lymphomas, even if the few cases analyzed for cell surface phenotype were $CD3^+$ LGLs of T cell origin (440). LGL infiltrates are observed in the red pulp only of the spleen and in the liver sinusoids, with minimal involvement of lymph nodes and no involvement of the thymus, i.e., following the typical localization of NK cells in healthy individuals (439–441).

A possible retroviral etiopathogenesis has been suggested in some cases of LGL lymphocytosis, because of the presence of high-titer antibodies to either human T lymphotropic virus type I (HTLV-I) or HTLV-II (441).

E. *In Vivo* Differentiation of NK Cells

In experimental animals there is evidence that NK cells originate and, at least in part, differentiate in the bone marrow. Treatment of mice with ^{89}Sr (a bone-seeking isotope) depresses splenic NK cell activity, but leaves CTL generation and macrophage-mediated cytotoxicity intact (442-444). Moreover, bone marrow reconstitution of radiation chimeras produced between pairs of histocompatible high and low NK cell-reactive mouse strains resulted in restoration of NK cell activity in the spleen. The chimeras were high or low NK cell reactive, depending on the bone marrow donor strain, and were independent of host environment (445). Similar experiments have been performed using the beige mouse strain presenting defective NK cell activity (446, 447). Radiation chimeras were used to demonstrate that NK cell activity was determined by the phenotype of the marrow donor and not by the genotype of the irradiated recipient (448), confirming that the generation of NK cells is an inborn and autonomous function of the bone marrow.

The role of the bone marrow as a necessary microenvironment for NK cell differentiation is further suggested by the failure of NK cell differentiation in congenital or 17β-estradiol-induced osteopetrotic mice (210, 414, 415). However, data obtained using osteopetrotic *mi/mi* mice must be interpreted cautiously: Heterozygous $^{+}/mi$ animals, which have no defect in the final bone formation, present a level of NK cell activity that is 50% of that of +/+ animals, suggesting the possibility of an effect of the *mi* locus on NK cells not mediated through osteopetrosis (449). In the estradiol-treated mice, NK1.1^{+} target-binding lymphocytes, which are noncytotoxic and not IFN inducible, are detectable. These cells might represent NK precursor cells preceding the marrow-dependent stage of NK cell differentiation (210). This NK-1.1 target-binding, nonlytic NK cell precursor was also observed in 8- to 9-day-old mice, before functional mature NK cells appear (210).

Data from irradiated patients and experimental animals suggest that mature NK cells might be relatively short-lived and radiation resistant (450-456). In mice sublethal total body irradiation induces a decrease in NK cell activity, beginning on day 14 after irradiation; the activity is fully restored, however, after 6-8 weeks (454). These data suggest that murine mature NK cells are relatively radioresistant, renewable cells with a life span of up to 2 weeks and that their direct progenitors are radiosensitive. Leukemogenic split-dose irradiation determines a more persistent depression of NK cell activity (453, 457). Similarly, in most irradiated

patients depression of NK cell activity is observed, with subsequent recovery after 3–4 months (450). The lack of recovery in some patients could be attributed either to the irradiation or the immunosuppressive protocol used or to the effect of the underlying malignant disease that prompted irradiation.

A different and probably more accurate evaluation of the life span of NK cells has been obtained by Miller (455), using the cell cycle-specific cytotoxic agent hydroxyurea (HU). Total NK cell activity of murine femoral marrow was unchanged for 10.5 hours after the first HU injection, indicative of the transition time for the DNA-synthesizing NK cell precursor to become an active NK cell. This was followed by an exponential decline with a half-time of 7.9 hours, reflecting the rapid exponential renewal of NK cells in the marrow. In the spleen total NK cell activity was unchanged for 20.5 hours, indicating the transit time from the last DNA synthesis of the precursor cells in the bone marrow to the appearance of the functional NK cells in the spleen, and then declined exponentially, with a half-time of 24.15 hours, suggesting a half-life of NK cells in the spleen of, on average, 1 day (455). These results have been extended by Pollack and Rosse (458), using [^{3}H]thymidine pulse-chase techniques *in vivo*. In these experiments, NK cells were identified by their ability to bind YAC-1 cells, after elimination of B cells. Two NK cell populations could be distinguished in the bone marrow: large proliferating target-binding cells (TBCs) (25% in S phase) and small postmitotic TBCs, probably derived from the large TBCs which, in turn, were derived from a more rapidly proliferating precursor population. Migration of labeled NK cells from the bone marrow to the spleen required at least 2 days; some of these cells in the spleen survived 2 months or longer, and little or no proliferation of NK cells occurred in the periphery of unstimulated mice (458).

The persistence of NK cells observed in the spleen in this study was much longer than that reported in the study by Miller (455). This discrepancy might reflect an underestimation of long-lived NK cells in the spleen in the latter study, since cells with reduced NK cell activity, which might characterize older NK cells, would not necessarily be detected in experiments based on measurement of residual cytotoxic activity. Analysis of renewal of NK cells using HU depletion experiments also showed that nude mice, with increased NK cell activity, have increased cell dynamics, involving proliferating precursor NK cells (459).

After bone marrow transplantation NK cells are the first lymphocyte population to reconstitute the recipient (460, 461). The pretransplant irradiation therapy does not immediately abrogate NK cell activity in the patients, but the ability of their cells to maintain cytotoxic activity

against K562 cells or to generate activity against tumor cells during culture in the presence of IL-2 is completely suppressed, suggesting a complete block in the proliferative ability of both NK cells and their precursors (462). After transplantation NK cells and LGLs appear, in both humans (463) and experimental animals (464), already at 1 week, and their number and activity increase to a peak at 30–50 days, slightly declining thereafter to normal or slightly subnormal levels (461,463). The appearance of NK cells precedes that of any other lymphocyte type, and at 20–30 days NK cells may represent 50–90% of all peripheral lymphocytes (465). The appearance of cytotoxic NK cells parallels that of IL-2-inducible cytotoxic cells: Both precursor and effector LAK cells in the patients are $CD3^-$ cells with an NK phenotype (462). The spontaneously cytotoxic NK cells in the recipient are activated, as suggested by their lymphoblastic appearance and by their ability to lyse efficiently not only K562 cells but also Daudi cells, tumor cells, and fresh leukemia cells (462, 464, 466). The $CD3^-$ NK cells obtained from transplanted leukemia patients and able to lyse leukemia cells have been cloned and shown by chromosomal analysis to derive from the donor bone marrow (466). Graft-versus-host disease (GVHD) in some cases has been associated with an accelerated appearance of cytotoxic NK cells following transplantation (467). The level of NK cell activity posttransplantion correlates inversely with the probability of CMV active infection (468). The establishment of active CMV infection has been shown to be followed by either a decrease (467) or an increase (463) in NK cell activity. These data confirm that NK cell precursors are contained in bone marrow and can rapidly proliferate and reconstitute the organisms.

The requirement for cell division during the maturation of NK cells after injection of bone marrow cells in irradiated mice was shown by the ability of irradiation or treatment with HU 7 days after bone marrow cell inoculation to prevent the appearance of normal NK cell levels (469). The replicating NK cells freshly derived from the bone marrow are $Thy\text{-}1^+$ but rapidly differentiate to $Thy\text{-}1^-$ cells, except in thymectomized mice, in which they remain $Thy\text{-}1^+$ (470, 471). The suppressor effect of the thymus on NK cell differentiation has also been shown in the low-NK cell activity mouse strain SJL; thymectomy of SJL mice as late as 25 days after birth increases the NK cell activity from low to intermediate levels (472).

The transplantable precursor cells for murine NK cells have been analyzed in detail by Hackett *et al.* (210, 412, 473). The bone marrow NK cell precursors were analyzed by transplantation in anti-asialo GM_1 antibody-injected mice and by detection of NK cell activity as the ability of the animals to clear intravenously injected labeled YAC-1 cells from

the lung (473). The bone marrow precursor cells were found to be NK-2.1$^-$, asialo-GM$_1$$^-$, Thy-1.2$^-$, Qa-5$^-$, Qa-2$^+$, and H-2$^+$ (473). Differentiation of the NK precursor cells *in vivo* requires an intact marrow microenvironment, because 17β-estradiol-treated mice fail to sustain NK cell differentiation (473). Bone marrow cells from *W/Wv* anemic mice and marrow from *scid* mice contain a normal frequency of NK cell precursors, indicating that the NK precursor cells are distinct from those of myeloid cells and of T and B lymphocytes, respectively (412, 473). In a similar system, i.e., transplantation of NK precursor cells in aged syngeneic mice with low splenic NK cell activity, if any, Miller (474) demonstrated that spleen cells but not fetal thymocytes, rich in prethymocytes, contain NK precursor cells. Thus, fetal thymic pre-T cells neither demonstrate nor develop NK cell activity. IL-1, IL-2, and IFN-α/β accelerate the reconstitution of irradiated mice by NK precursor cells (475–477). However, IFN at 14 days after transplantation induces a significant suppression of NK cell activity due to the induction of suppressor cells (476). Treatment of recipient animals with IL-3 determined a reduced appearance of NK cell activity and cells with NK cell phenotypic markers (477).

F. *In Vitro* Models of NK Cell Differentiation

The availability of culture systems for the analysis of NK cell differentiation could allow a more precise identification of the precursor cells and of the cellular and humoral interactions that are required for differentiation-maturation. As discussed in detail in Section V, one of the difficulties of these studies is that most mature terminally differentiated NK cells can be rapidly induced into the cell cycle and can continue proliferating *in vitro* in the presence of IL-2 for several weeks. Any system that analyzes *in vitro* differentiation of NK cells should clearly distinguish between the differentiation of NK cell precursors and the induced proliferation of mature resting NK cells. Recently, various experimental systems have been described that strongly suggest the *in vitro* differentiation of NK cell precursor cells from bone marrow and peripheral blood. Unlike bone marrow and peripheral blood, cultures of thymocytes seem to generate mostly non-MHC-restricted CTLs.

1. Bone Marrow

The original studies of NK cell differentiation *in vitro* using murine bone marrow and other organs were difficult due to the lack of well-characterized and monospecific reagents. Using alloantisera against NK-1.1 antigen, Koo *et al.* (478, 479) identified NK-1.1$^-$ and NK-1.1$^+$ NK cell precursors devoid of cytotoxic activity, but their observation was

weakened by the reactivity of the alloantisera with a large proportion of immature hematopoietic cells. The availability of better reagents, including monoclonal antibodies against NK-1.1 (209), and of purified or recombinant lymphokines now allows a more detailed analysis of NK cell differentiation *in vitro*. IL-2 or IL-2-containing conditioned medium allows the generation of cytotoxic NK cells from murine bone marrow cultures depleted of mature NK cells by treatment with antibodies or with 5-fluorouracil, which is selectively toxic for differentiated cells (480–483). The cytotoxic cells generated in these culture systems have, at least in part, the phenotype of mature NK cells, including expression of asialo-GM_1 and, often, Thy-1 and NK-1.1 (481–483). The precursor cells are asialo-GM_1^- but usually Thy-1^+ (482, 483). The NK precursor cells detected in these *in vitro* systems might therefore be more mature than the NK progenitor cells detected by adoptive transfer *in vivo*, which are asialo-GM_1^-, Thy-1^-. In the absence of IL-2, differentiation of NK cells from bone marrow cells was not induced by IL-1, IL-3, or IFN-α/β (481). However, IFN and IL-1 (or hemopoietin 1) are able to synergize with IL-2 in inducing differentiation (483–486). A similar effect was observed with TNF and lymphotoxin (486). On the contrary, IL-3 (487), transforming growth factor-β (TGF-β), IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (486) significantly inhibit NK cell differentiation. Epidermal growth factor and fibroblast growth factor have no effect (486).

Functionally active NK cells are no longer detectable by 1 week of culture in cultured murine bone marrow harvested from Dexter-type long-term marrow cultures (488). IFN does not induce cytotoxic NK cells in these cultures. However, up to 9 weeks of culture, cells cytotoxic for YAC-1 target cells could be generated after 1 week in secondary cultures in the presence of IL-2-containing conditioned medium (488). Like NK cells, the cytotoxic cells were asialo-GM_1^+, Thy-1^+, Ly-5^+, NK-1^+, Ly-1^-, but, unlike NK cells, $\sim 30\%$ of the cells expressed Ly-2 antigen (488).

Studies using human bone marrow (489, 490) have shown that IL-2 induces a proliferation-dependent generation of cells cytotoxic for K562 targets that are $CD3^-$ NKH-1/Leu-19^+ and, in part, $CD16^+$ cells. A detailed analysis of the precursor cells in these cultures has not yet been presented.

2. *Peripheral Blood*

Numerous studies have analyzed the activation of peripheral blood NK cells and the induction of their proliferation by biological response modifiers such as IL-2 or IFN. However, few attempts have been made

to distinguish the differentiation of precursor cells from the proliferation of mature resting NK cells. PBLs treated with the lysosomotropic agent L-leucine methyl ester (LeuOMe) are specifically depleted of $CD16^+$, $CD11b^+$, HNK-1/Leu-7^+ cells. LeuOMe-treated PBLs were not responsive to IFN, but regenerated NK cell activity after treatment with IL-2 or stimulation in mixed-leukocyte culture (491). The precursor cells of the cytotoxic NK cells are not granular, but are of the same low density as mature NK cells (large agranular lymphocytes). Generation of NK cells with LGL morphology from high-density small lymphocytes of $CD3^-$, $CD2^+$, $CD11b^+$ phenotype has been demonstrated by culturing the lymphocytes in the presence of mitomycin C-treated autologous T blasts and IL-2-containing conditioned medium (492, 493).

3. Thymus

Early studies showed that IL-2 induces human thymocytes to bind and lyse K562 target cells (494, 495). Cytotoxic activity is not present in fresh thymocytes, appears after 3-day culture in the presence of IL-2, and reaches a maximum at day 7 (496). All of the cytotoxic cells are NKH-1/Leu-19^+ (496–498) and granular, as shown by staining with the lysosomotropic vital dye quinacrine (498). Although one of the original studies reported the presence of FcγR on the effector cells (494), the presence of CD16 antigens has never been detected in the thymocyte cultures (498). The majority of NKH-1/Leu-19^+ cells in cultured thymocytes are also $CD3^+$, but a significant proportion of NKH-1/Leu-19^+, $CD3^-$ cytotoxic cells is always present (496, 498). Because both $CD3^+$ and $CD3^-$ thymus-derived, non-MHC-restricted cytotoxic cells do not express CD16, and because NKH-1 and CD3 are often coexpressed in non-MHC-restricted T cell clones, it is difficult to identify whether the $CD3^-$ cells are T or NK cells. Because the thymus has been shown not to contain NK cell progenitors in adoptive transfer experiments, the possibility should be considered that the $CD3^-$ cells represent expansion/activation of T cells at an immature stage of development, before functional expression of the TCR on these cells.

V. Activation and Effector Mechanisms of NK Cells

When NK cells leave the bone marrow, they revert to a resting state and all or most circulating or tissue NK cells are noncycling. NK cells are short-lived in the peripheral blood and in spleen, but it is as yet unknown how long tissue-associated NK cells persist. The most striking characteristic of NK cells is that resting circulating NK cells, present at all times in all healthy individuals, are "natural" functionally active cells, i.e., they can be triggered to lyse a target cell within minutes when

confronted with the appropriate target structure or with an antibody-coated target cell. Other NK cell functions, such as lymphokine production and the regulation of hematopoietic and adaptive immune cells, are also mediated by resting NK cells. This ability of NK cells to respond to a triggering stimulus without the need for preactivation enables them to participate in the first line of defense against various pathogens. In this respect NK cells resemble other effector cell types of nonadaptive immunity such as granulocytes and monocyte/macrophages. Moreover, the functional activity of NK cells, like that of other nonadaptive effector cells, is rapidly enhanced by cytokines such as IFN and IL-2. This modulation of NK cell functional activity does not require cell division. *In vivo*, however, conditions such as virus infection or a strong antigenic stimulus induce both the activation of NK cells and an increase in NK cell number, due to increased proliferation, probably mostly at the bone marrow level. This *in vivo* response is maximal at 3–4 days, before adaptive immune responses become effective, and is reminiscent of the myelopoietic reaction to bacterial infection. Unlike myelomonocytic cells, differentiated resting NK cells and also resting T and B lymphocytes can be rapidly induced into the cell cycle and maintained *in vitro* in a proliferative state, for 30 or more cell divisions in a 2- to 3-month period. The *in vivo* proliferative response of NK cells is likely to be contributed by both the centralized proliferation of NK progenitor cells in the bone marrow and the induction of circulating NK cells into the cell cycle.

As illustrated in Fig. 6 and detailed in this chapter, the response of NK cells to an external stimulus can be divided into three sequential phases. In the first phase interaction of NK cells with target cells or with immune complexes induces a rapid response (1–10 minutes) associated with cytotoxicity and the release of granule contents. These same interactions and also stimulation by IL-2 induce (10 minutes to 2 hours), independently and synergistically, the second phase, in which genes encoding lymphokines and surface activation antigens, including the p55 chain of the IL-2 receptor (CD25 antigen), are transcribed and expressed. In the presence of IL-2, the NK cells proceed into the third phase (1–3 days) of the response, with blast formation, DNA synthesis, and proliferation. The various stimuli and modulating factors affect these three phases of the NK cell response differently, and the role of each phase in the various *in vivo* and *in vitro* functions of NK cells differs.

A. Sensitivity of Target Cells to NK Cell-Mediated Killing

There is considerable variability in the sensitivity of different cell lines and fresh tumor or normal cells to the cytotoxicity mediated by NK cells. The recognition structure on NK cells has not been identified, but the studies described below suggest the possibility that more than one single

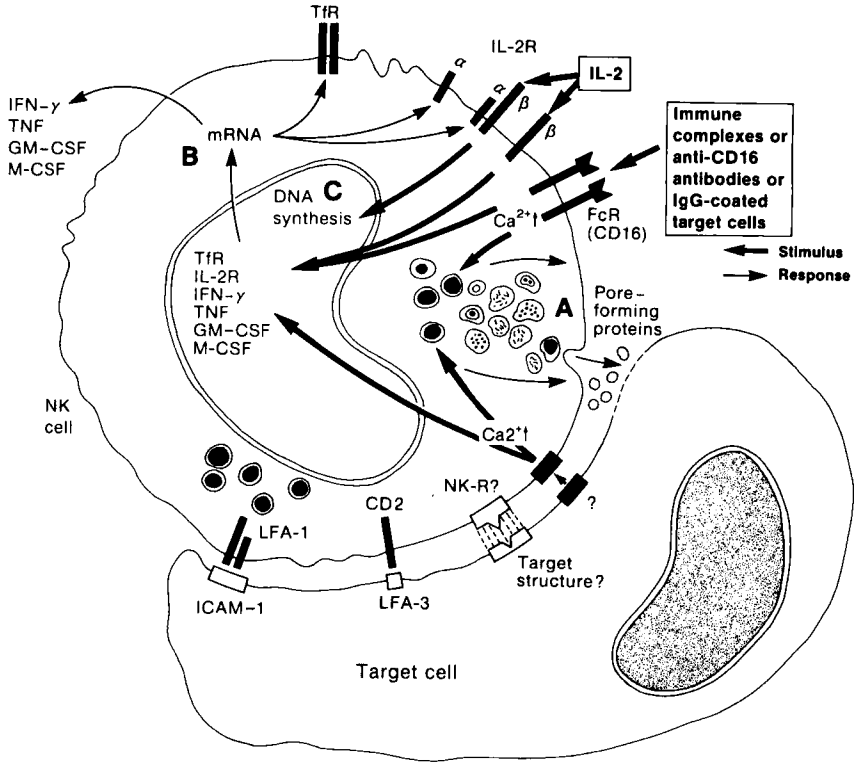


FIG. 6. Model of NK cell activation following interaction with target cells or immune complexes. The interaction of NK cells with target cells involves unknown receptor(s) responsible for binding and NK cell activation (signal transduction). Signal transduction involves enhanced phosphoinositide turnover and increase of $[Ca^{2+}]_i$, due to release of Ca^{2+} from intracellular stores and influx of extracellular Ca^{2+}, observed upon interaction of NK cells with NK cell-sensitive target cells. Similar signal transduction mechanisms are activated during interaction of CD16 FcR with ligands, i.e., with anti-CD16 antibodies, immune complexes, or IgG-coated target cells. Three types of response are observed: (A) activation of the cytotoxic mechanism, with morphological alteration and secretion of the content of granules, including cytotoxic molecules such as pore-forming proteins, NKCF, and others; (B) transcription of lymphokine and cell surface receptor genes and expression of their products, with a synergistic induction mediated by IL-2; (C) proliferation of NK cells, mostly induced by IL-2 interaction with the high-affinity IL-2 receptor (IL-2R) [p75 (β) and p55 (α) chain dimer] or with the p75 chain (β) of the IL-2 receptor, but modulated by the regulatory effect of NK-target cell or FcR (CD16)-ligand interactions on the expression of the IL-2 receptor [p55 chain (α) or CD25 antigen] gene. These mechanisms are discussed in detail in the text.

structure is involved and that different target cells might be recognized through different structures. One general characteristic of NK cell-mediated killing is that cells from the homologous species are usually killed more efficiently than are heterologous cells; however, this phenomenon has been rarely studied and remains unexplained (499–501).

The prototype target cell lines used in each species, the K562 cell line for human NK cells and the YAC-1 cell line for mouse and rat NK cells, are among the most sensitive cell lines in each system. However, almost any cell is sensitive to a certain extent to NK cells, if the concentration of effector cells is sufficiently high or if the NK cells are activated by IFN or IL-2. When evaluating the sensitivity of target cells to lysis, it should be considered that several different factors play a role in determining cell lysis. The ability of a cell line to bind to NK cells is necessary but not sufficient to render it sensitive to lysis (502). In order to activate the cytotoxic mechanism in the NK cells, a structure on the target cells, possibly distinct from the one responsible for cell binding, must trigger the effector cells (503, 504). This second requirement can be circumvented if the target cells present molecules that can interact directly with functional receptors on the NK cell surface, such as: (1) IgG antibodies, binding to CD16 (ADCC); (2) C3, presumably binding to CD11b (C3bi receptor) (505, 506); (3) antibodies to CD16 or CD2 antigens on NK cells and binding with the Fc fragment to the FcR on target cells (reverse ADCC) (507); and (4) heterocross-linked antibodies that recognize an NK cell receptor (e.g., CD16) and an antigen on the target cells (508, 509). When target cells are bound to NK cells and the lytic mechanism is activated, lysis of the target cells still depends on the intrinsic sensitivity of the target cells to the lytic mechanism. Certain types of target cells may activate the cytotoxic ability of NK cells and therefore might appear to be very sensitive to NK cells. For example, NK cell activation is observed with target cells infected with viruses or mycoplasmas (79–82), but may require the participation of accessory cells and IFN (510), and is characterized by an increase of the rate of lysis during the cytotoxic assay (see Fig. 3).

The intrinsic susceptibility of target cells to NK cell lysis appears to be dependent on components of the cell membrane. Glycoproteins isolated from the target cell membrane and inserted into artificial membranes were able to inhibit conjugate formation between human or rat NK cells with their target cells in a species-specific manner (500). However, cytotoxicity was not inhibited in these experiments (500), raising the possibility that lytic factors acting at short range but not requiring cell contact might play a role in NK cell-mediated cytotoxicity. The species specificity of the inhibition suggests that the species specificity

of the NK cell cytotoxicity is at the level of NK-target cell conjugate formation. If liposomes containing membrane components from sensitive target cells are fused with resistant target cells, the resistant target cells become sensitive to NK cell-mediated lysis (501). In this experimental system (501) NK cells did not bind to or lyse heterologous target cells even when fused with liposomes containing membrane components from homologous, sensitive target cells. Moreover, liposomes containing membrane components from heterologous NK cell-sensitive target cells could not confer NK cell sensitivity to homologous NK cell-resistant target cells. These results suggest that species restriction might be at both the recognition (binding) and triggering levels.

Although NK cell lysis is often defined as specific for tumor and virus-infected targets, these cytotoxic cells can also lyse normal cells, and often it is difficult to demonstrate lysis of malignant cells. In most cases, virus-infected cells are no more sensitive than uninfected cells but are lysed through mechanisms that involve activation of NK cells (79, 511-513). Lysis of freshly obtained tumor target cells can be demonstrated using both autologous and allogeneic NK cells, but often procedures such as enrichment or stimulation with IFN or IL-2 must be used before significant killing is observed (514-517). In some cases tumor cells freshly obtained from the patients are insensitive to NK cell-mediated lysis; however, brief (24-hour) incubation *in vitro* in the case of acute myeloid leukemia blasts (518) or treatment with anti-Ig antibodies in the case of B cell chronic leukemia cells (519) render these cells sensitive to NK cell lysis. YAC-1 cells, when grown *in vivo* and directly obtained from the animals, are also resistant to lysis unless cultured for a few days (520). In this case the NK cell sensitivity increases during *in vitro* culture concomitant with a decrease of H-2 antigen expression, suggesting that an effect of IFN *in vivo*, as discussed below, might be responsible for NK cell resistance (521). Experiments using IL-2-activated NK cells against autologous endometrial carcinoma cells and normal endometrial epithelium cells have shown that the carcinoma cells were lysed more efficiently than were the normal cells, suggesting that activated NK cells might, at least for this type of tumor, selectively lyse the malignant cells (522). In several studies, transfection with the *ras* oncogenes has been shown to render cells sensitive to NK cell lysis (523-525), although the lack of correlation of sensitivity to lysis with transformation (525) makes it difficult to conclude from these observations that NK cell sensitivity arises during the early stages of cellular transformation. However, Nabi *et al.* (526) showed that suppression of some characteristics of transformed cells, such as lack of contact inhibition, renders human malignant target cells resistant to NK cell lysis.

The possibility that the sensitivity of transformed cells to NK cell lysis is determined by their high proliferation rate and by structures expressed at particular stages of the cell cycle was excluded by studies showing that susceptibility to lysis is independent of the cell cycle stage (527, 528).

Kiessling and Wigzell (529) proposed that the function of NK cells was the surveillance of primitive cells, since embryonic thymus and bone marrow contain NK-sensitive cells (36), NK cells lyse undifferentiated but not differentiated embryonic carcinoma cells (530), and induction of differentiation of the K562 (531, 532) and U937 (529) cell lines reduces their sensitivity to NK cells. Evidence against this theory comes from the observation that NK-deficient beige mice are no more susceptible than are normal mice to the growth of experimental embryo-induced teratoma and teratocarcinoma (533). Also, phorbol diester treatment of K562 cells, which was originally shown to induce differentiation and decrease sensitivity to NK cell lysis (531, 532), was subsequently reported to increase sensitivity (534) or to have opposite effects on different subclones of K562 (535). Phorbol diesters induce NK cell sensitivity in many cell lines (534, 536), possibly by inducing a decrease in cell surface sialic acid content (534, 537). That autologous or allogeneic NK cells or activated NK cells can lyse normal differentiated cells is supported by the relative sensitivity of normal fibroblast strains (34, 35) and normal fresh monocytes to NK cell lysis (538, 539).

Several experimental observations suggest an inverse correlation between expression of class I MHC antigen on target cells and sensitivity to NK cell lysis. Differentiation of teratocarcinoma cells and of normal thymocytes results in increased H-2 expression and decreased sensitivity to lysis (530, 540). In the YAC-1 and other cell lines, low expression of class I antigens correlates with high sensitivity to NK cells and limited growth potential *in vivo*, whereas variants with high class I expression are resistant to NK cells and highly metastatic *in vivo* (541–546). IFN treatment of target cells determines both resistance to NK cell lysis and increased expression of class I MHC antigens (543, 544, 546). However, other studies failed to demonstrate an absolute correlation between class I MHC expression and sensitivity to NK cell lysis (547, 548), including two studies (549, 550) in which transfection and expression of H-2D or H-2K genes in target cells were shown to have no influence on NK cell susceptibility.

Another example of dissociation between class I MHC antigen expression and NK cell sensitivity comes from adenovirus-transformed cells: When the *E1A* gene from adenovirus 5 was present, transformed cells had little or no class I MHC antigen expression and were resistant to NK cell lysis and tumorigenic, whereas when the *E1A* gene from

adenovirus 12 was present, transformed cells expressed high levels of class I MHC antigens and were sensitive to NK cells and poorly tumorigenic (551). Overall studies suggest that in some cases class I MHC antigen expression prevents the triggering of NK cells and, as suggested in one study (545), the formation of NK-target cell conjugates; however, in other cases this negative control is ineffective, possibly because other structures are present on the target cell membrane and are recognized by NK cells.

Several antigens present on target cell membranes have been proposed as possible NK cell target structures. The data are largely contrasting, and evidence in favor of the role of a single molecule has not been confirmed in other systems. It is possible that NK cells recognize different molecules that either play a primary role as target molecules responsible for conjugate formation and/or triggering or exert an accessory but not essential role in increasing the binding affinity between NK and target cells. The transferrin receptor (TfR) has received much attention as a possible target antigen on the basis of inhibition by anti-TfR antibodies and correlation between TfR expression on target cells and sensitivity to NK cells or ability of the cells to compete (552–554). However, in several other studies (533, 555, 556) these results could not be reproduced, indicating that the role, if any, of the TfR as a target cell antigen is not unique. One study (557) suggested that the presence of either TfR or CDw32 FcγRII on cell lines could be sufficient for NK cell recognition, on the basis of antibody inhibition analysis. Expression of the CD15 antigen (3-fucosyl-*N*-acetyl-lactosamine hapten or X-hapten) on cell lines has been correlated with binding to and lysis by NK cells (558). Those findings, together with the ability of anti-CD15 antibody to inhibit NK cell-mediated lysis (559), suggested a role for the CD15 hapten in NK cell killing. Other investigators (143) identified the 140-kDa heterodimer detected by antibody 4F2 as the target cell structure recognized by several non-MHC-restricted cytotoxic T cell clones that express an identical TCR idiotype and recognize target cells through the TCR in an antigen-specific fashion; however, no role for 4F2 antigen was found in the killing mediated by human peripheral blood $CD3^-$, TCR^- NK cells (554, 560). Recently, it was found that monoclonal antibodies directed against a 42-kDa molecule, possibly existing as a homodimer on target cells, efficiently inhibit NK cell binding to and lysis of all human and mouse target cell lines tested and of the fish parasite *Tetrahymena pyriformis*, suggesting that the antigen is a primitive recognition structure present on the target cells (561).

Several reports have described the ability of simple or complex sugars to inhibit NK cell-mediated lysis (562–564). The inhibition was observed

only at high sugar concentrations (>50 m*M*), but hexose phosphates at less than 25 m*M* were inhibitory (562) and hexose 6-*O*-sulfate esters such as mannose-6-sulfate or galactose-6-sulfate were inhibitory at 1–2 m*M* concentrations (564). Although it is known that sugars act at a postbinding stage (after the Ca^{2+}-requiring step) to inhibit lysis, the mechanism of this inhibition remains unclear (563, 564). The ability of certain glycopeptides from the target cell membrane to inhibit NK cell-mediated lysis suggests the requirement for target cell expression of certain carbohydrate structures (565). However, several lines of experimental evidence have excluded a role for the mannose-6-phosphate receptor on either the effector or target cell surface in NK cell-mediated lysis (566, 567). A possible role for lectinlike substances has been proposed on the basis of inhibition of pig NK cells by lectin-specific antibodies (568).

IFN, a potent activator of NK cell cytotoxic activity, antagonistically protects target cells from NK cell lysis (34, 503, 569, 570). These antagonistic effects of IFN may play a major regulatory role in *in vivo* NK cell activity, as discussed further in Section IX. Treatment of several cell lines for a few hours with IFN-α, -β, or -γ induces a dose-dependent inhibition of target cell sensitivity to NK cell lysis (34, 503, 569, 571). Lysis by complement, ADCC, or CTLs is not affected (503, 569, 570, 572). The induction of resistance requires active RNA and protein synthesis in the target cells (34). After 24–48 hours' incubation in the absence of IFN, target cells regain their sensitivity to NK cell lysis (503). The inhibition of NK cell lysis by IFN treatment of target cells is at a postbinding stage, as indicated by the observations that IFN-treated target cells are able to form conjugates with NK cells (503, 570) but not to induce NK cell cytotoxic factor (NKCF) release (573, 574) or Ca^{2+} influx in the effector cells (575). However, IFN-treated cold target cells fail to compete for the lysis of untreated ^{51}Cr-labeled target cells (503, 570), showing that competition experiments measure the functional interaction of NK cells with target cells and not only target cell recognition (76). IFN-treated target cells are still sensitive to NK cell lysis triggered by IgG antibodies in ADCC (503, 569), and to the lysis mediated by NKCF (573, 574). Surprisingly, however, sensitivity of IFN-treated K562 cells to the lysis mediated by purified granule material was reduced (575). These contrasting results might reflect the use of different target cell types, i.e., normal fibroblast strains or K562 cells, in the various studies.

Different cell lines differ greatly in their sensitivity to the NK cell-protecting effect of IFN, and a correlation has been found between the abilities of IFN to induce antiviral activity and to protect the target cells from NK cell lysis (34, 569). A few units of IFN-α completely protected

fibroblasts from lysis, whereas even very high concentrations of IFN-α or -γ induced only partial protection of K562 cells (34, 576). Transformed or tumor-derived cell lines, on average, are more sensitive than are normal fibroblasts to IFN, although individual tumor-derived cell lines display high or intermediate sensitivity to the protective effect of IFN (569). Infection of target cells with most viruses completely prevents the ability of IFN to protect target cells, probably because lytic infection by these viruses suppresses host cell RNA and protein synthesis (34). However, viruses such as lymphocyte choriomeningitis virus that do not suppress RNA and protein synthesis do not prevent the protective effect of IFN on target cells (511). In addition to fibroblasts, other normal cells are protected by IFN against NK cells, including normal thymocytes (521) and monocytes (577). The IFN-induced increase in sialic acid expression on target cells has been inversely correlated with sensitivity to NK cell lysis (578). The abilities of IFN to increase class I MHC expression and to decrease sensitivity to NK cells have been considered as evidence for the preferential NK cell lysis of target cells with low class I MHC expression (543, 544, 546); however, the demonstration that IFN can protect target cells without inducing class I expression (579) has shown that this is not the major mechanism by which IFN protects target cells. Normal and tumor target cells are rendered resistant to NK cell lysis by *in vivo* exposure to IFN (521, 570), and IFN-treated B16 melanoma cells become NK cell resistant *in vitro*, with increased metastatic potential *in vivo* (580). The antagonistic effects of IFN on NK cells and their target cells *in vivo* may render NK cells selective against virus-infected target cells or IFN-resistant malignant cells by protecting normal cells from NK cell lysis and from competition with sensitive target cells. However, malignant cells that maintain IFN sensitivity or viruses that do not induce IFN resistance in the host cells might be able to escape the surveillance mechanism of NK cells.

B. Receptors Involved in NK Effector-Target Cell Interaction and Signal Transduction Mechanisms

With the exception of the CD16 FcγR used in ADCC, there is no definitive information yet on the type of receptor used by NK cells for target cell recognition and killing. TCR genes are not rearranged and TCR proteins are not expressed on peripheral blood $CD3^-$ NK cells, although TCR$\alpha\beta^+$ and possibly most TCR$\gamma\delta^+$, non-MHC-restricted CTLs may use their TCR for target cell recognition (142, 202-204).

Experiments of target cell cross-competition served to define several cross-competing target cell groups (13, 581, 582), suggesting some selectivity in the specificity of NK cells. Studies of the specificity of IL-2-grown

NK cells have indicated heterogeneity in the range of target cells lysed by the clones but have not demonstrated a clonally distributed specificity of NK cells (20, 583, 584). Recently, TCR^-, $CD3^-$ NK cell clones, originated from mixed-leukocyte cultures with lymphocytes from some but not all donors, were shown to specifically lyse allogeneic cells bearing the stimulating alloantigens (585). The molecular basis of this phenomenon is not known and may involve preferential growth in the mixed cultures of NK cells with a single receptor or a combination of receptors that preferentially recognize non-MHC-encoded polymorphic structures on the stimulator cells.

It was hypothesized that, at least in some experimental systems, NK cell activity depends on the presence of natural cytophilic antibodies bound *in vivo* to the FcγR and directed against target cell surface antigens (586-588). Although this mechanism may play a role in some systems, it is clearly not a general mechanism for NK cell-mediated cytotoxicity, as shown by several lines of evidence: (1) anti-IgG or anti-CD16 FcγR antibodies inhibit ADCC but not NK cell killing (79, 114, 132, 589-591), (2) phorbol diesters induce down-modulation of CD16 FcγR and inhibition of ADCC but not of NK cell killing (133), and (3) NK cells from *scid* mice and from several SCID patients are cytotoxic both *in vitro* and *in vivo* even if the animals and the patients do not produce IgG (400, 401, 410, 412, 413). However, interaction of CD16 FcγR with aggregated IgG, immune complexes, or cross-linked monoclonal anti-CD16 antibodies at 37°C induces inhibition of both ADCC and spontaneous cytotoxic activity (32, 132, 592-594). These data suggest that CD16 FcγR are not directly involved in the mechanism of NK cell-mediated spontaneous cytotoxicity, but that aggregation of the FcγR, e.g., by interaction with IgG-sensitized target cells or with immune complexes, may trigger and eventually exhaust the same cytotoxic mechanism involved in spontaneous cytotoxicity.

Like the inactivation of NK cells upon interaction with IgG-coated target cells, the interaction with NK cell-sensitive target cells also induces inactivity of NK cells, resulting in a decrease in cytotoxic rate with time of incubation (76). When PBLs containing NK cells were incubated at 37°C, but not at 4°C, with K562 target cells, NK cells were completely unable to lyse freshly added target cells after 4 hours of incubation (76). This inactivation was not seen with target cells, such as mycoplasma- or virus-infected cells, that were able to induce IFN production (76, 595), and IFN (76) and IL-2 (596) restored cytotoxic ability in NK cells that were separated from the target cells. The inactivation of NK cells was not target cell specific, and NK cells also showed abrogated or reduced cytotoxicity against target cells unrelated to those used for inactivation

or against IgG-coated target cells (76, 597). IFN-treated target cells, which were resistant to NK cell lysis, were unable to induce NK cell inactivation (503, 595). These data were originally interpreted by assuming that after one or a few lytic interactions with the target cells, NK cells exhausted preformed lytic mediators required for both ADCC and spontaneous cytotoxicity and therefore became unable to mediate additional cytotoxicity unless stimulated by IFN or IL-2 (76). However, Brahmi *et al.* (598) demonstrated that the target cell-induced NK cell inactivation also occurs in the absence of Ca^{2+}, suggesting that it affects an early calcium-independent event in the activation of the human NK cell cytolytic mechanism.

Many studies have shown that interaction of NK cells with NK cell-sensitive target cells stimulate phosphoinositide turnover with production of the Ca^{2+}-mobilizing messengers inositol trisphosphate (IP3) and IP4 (599–602; M. Cassatella and G. Trinchieri, unpublished observations). Ca^{2+} influx in the effector cells has been suggested on the basis of ^{45}Ca uptake data (575, 603), although uptake by effector and target cells could not be distinguished by those experimental procedures. Recently, Windebank *et al.* (604), using liquorin-loaded NK cells, and we (M. Cassatella and G. Trinchieri, unpublished observations), using Fura-2-loaded NK cells, have shown that interaction of NK cells with target cells induces an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that is approximately proportional to the sensitivity of the target cell to lysis. The increase in $[Ca^{2+}]_i$ upon interaction with the target cell depends on both the release of Ca^{2+} from intracellular stores and the uptake of extracellular Ca^{2+} (M. Cassatella and G. Trinchieri, unpublished observations). The stimulation of NK cell phosphoinositide metabolism by target cells has been shown to require extracellular Ca^{2+} in one study (602) but not in others (601; M. Cassatella and G. Trinchieri, unpublished observations). These contrasting results are not surprising because chelation of extracellular Ca^{2+}, depending on the experimental conditions, may result in a slower depletion of $[Ca^{2+}]_i$, which eventually blocks phosphoinositide metabolism by preventing activation of the Ca^{2+}-dependent phospholipase C. Cross-linking of the CD16 FcγR by antibodies, immune complexes, or IgG-coated target cells also induces formation of IP3 and IP4 that does not depend on the presence of extracellular Ca^{2+} (601; M. Cassatella and G. Trinchieri, unpublished observations) and an increase in $[Ca^{2+}]_i$ (601).

In analogy with the data on activation of T cells through the CD3 complex (605, 606), it is possible to postulate that activation of NK cells through either CD16 FcγR or the receptor(s) for target cells induces activation of the same G protein, with subsequent induction of IP3 and

IP4 and increased $[Ca^{2+}]_i$. Following this activation, again by analogy with T cells (605, 606), the G protein may remain in a refractory state, thereby preventing activation of NK cells by either IgG-coated or sensitive target cells. The independence from extracellular Ca^{2+} of the stimulation of phosphoinositide metabolism in NK cells suggests that inactivation of the G protein, like the inactivation of the cytotoxic mechanism by target cells (598), should also not require extracellular Ca^{2+}.

The surface molecules of the CD11/CD18 family appear to play important functional roles in NK cell killing. Patients with severe deficiency of the common CD18 chain and therefore lacking all three molecules are deficient in NK cell activity (378, 607). Antibodies to the CD18 chain efficiently block NK cell-mediated cytotoxicity by preventing binding of NK cells to target cells (179, 608, 609). Patients with selective deficiency in CD11a (LFA-1) expression are also deficient in NK cell-mediated cytotoxicity (608). A series of antibodies directed against various epitopes of the CD11a molecule inhibited both NK cell-mediated and CTL-mediated cytotoxicity; these antibodies had different efficiency in inhibiting cytotoxicity, but the same hierarchy of functionally relevant CD11a epitopes was shown for NK cells and CTLs (608). The antibodies inhibited lysis at the effector cell level by preventing NK-target cell conjugate formation (608). When peripheral blood NK cells were tested, inhibition by anti-CD11a antibodies was observed with several different target cell lines (610); with one NK cell clone used as effector cells, lysis of K562 cells but not that of other target cells was inhibited (610), whereas with other clones, the opposite result was observed (611). CD11a (LFA-1) is therefore an important adhesion molecule in the interaction of cytotoxic cells, both NK cells and CTLs, with target cells; the variable requirements for the CD11a molecules in binding of different target cells from different clones suggest that that either CD11a is one of several receptors that NK cells can use for binding or it has only an accessory function, essential only when other receptors responsible for the specificity of the binding do not ensure a binding of sufficient affinity. No evidence has been provided that CD11a is a functional receptor capable of signal transduction and triggering of NK cells.

A possible role for the CD11b molecules, or receptor for the C3bi fragment (CR3), in NK cell-mediated cytotoxicity is suggested by the increased efficiency of NK cell lysis of Raji target cells when these cells bind C3 through their CR2 (505, 506). Expression of CD11b on the NK cells is required for the enhancing effect (612); however, it is possible that this phenomenon reflects only a bridging effect that enhances NK-target cell contact, without triggering of the NK cells through the CD11b molecule. Antibodies to CD11b or CD11c did not directly affect

NK cell-mediated cytotoxicity against a variety of target cells, including the Raji cell line, in the absence of complement (179), although the lysis of Raji cells with bound C3 was inhibited by anti-CDllb antibodies (612).

Antibodies against non-lineage-restricted epitopes of the T200 molecule (CD45) inhibit NK cell-mediated lysis but not CTL-mediated lysis, acting at the effector cell level at a postbinding stage (613, 614). Some antibodies blocked lysis of a wide range of target cells (614), but antibody 13.1 blocked lysis of K562 cells but not of T cell lines, even when clones able to lyse both types of target cells were used as effector cells (613). Antibody 9.1C3, which binds a protein dimer of 66 and 76 kDa that is associated with the T200 molecule, also blocked NK cell-mediated lysis at the effector cell level and at a postbinding phase (615). A rabbit antiidiotypic antiserum generated against antibody 9.1C3 was highly reactive with K562 cells, precipitating two molecules of 94 and 79 kDa, and inhibited NK-mediated lysis of K562 cells at a postbinding stage (616). Thus, T200, or molecules associated with it, appear to interact with cell surface molecules on at least some target cell types, creating a secondary postbinding NK-target cell interaction; however, the functional significance of this secondary interaction is unknown.

Laminin or its receptor represents another adherence system that plays a major role in NK cell killing (617). Rodent and human NK cells, more than other lymphocyte types, express a lamininlike structure (48 kDa on rat cells and 38 kDa on human cells) that is recognized by antilaminin antibodies (618–620). The 48-kDa molecule on rat NK cells is translated from a 2.4-kb mRNA homologous to part of the 8-kb mRNA encoding the β_2 subunit of laminin (617). Laminin and $F(ab')_2$ antilaminin antibodies block cytotoxicity at a postbinding stage, without inhibiting NK-target cell interaction (619, 620). The sensitivity of murine target cells to NK cell-mediated lysis has been correlated with the ability of NK cells to bind laminin (618). Expression of lamininlike molecules on the NK cell surface increases upon stimulation with IL-2 (620). Thus, it is possible that a lamininlike molecule on the NK cell after conjugate formation acts in continuing the lytic mechanism by binding to the target cells either through laminin receptors or to matrix laminin expressed by the target cells (620).

C-reactive protein (CRP) is expressed on a proportion of PBLs (621). The CRP present on PBLs is not bound to the CRP receptor; it is present in monomeric form and not in the pentameric native form, and is presumably produced by the lymphocytes (622). Earlier studies using antibodies against native CRP detected expression of CRP on 3–4% of the PBLs, and all of the positive cells had the phenotype of NK cells (621); however, using antibodies specific for determinants expressed only in the monomeric form, 20–30% of PBLs expressed CRP, suggesting

that cells other than NK cells might express it (622). Anti-CRP $F(ab')_2$ antibodies reduced NK cell function at a postbinding stage (623), suggesting that CRP might be another of the molecules involved in the secondary interaction between NK and target cells, or that anti-CRP antibodies act directly on effector cells by preventing NK cell triggering.

The CD2 E-R antigen expresses at least three distinct epitopes: Tll_1, the erythrocyte binding site; Tll_2, an epitope unrelated to the binding site but with the same cellular distribution of Tll_1; and Tll_3, an epitope expressed only in activated cells or in cells treated with anti-Tll_2 antibodies (624). CD2 is an antigen-independent pathway of T cell activation, and treatment of T cells with anti-Tll_2 plus anti-Tll_3 antibodies induces expression of IL-2 receptor, secretion of IL-2, and proliferation of T cells (624). Treatment of peripheral blood NK cells with anti-Tll_2 and anti-Tll_3 does not induce cell proliferation (625). This lack of a proliferative effect of anti-CD2 antibodies is probably due to a lack of IL-2 production (626), because anti-CD2 antibodies stimulated expression of the IL-2 receptor in NK cell clones (627) and increased the cytotoxic activity of both fresh NK cells (628) and NK cell clones (629, 630). Anti-CD2-treated NK cells showed increased adhesion to the target cells and oriented discharge of granules on the area of contact with the target cells (630); simultaneous treatment with anti-CD11a antibodies blocked NK–target cell adhesion and induced NK cells to secrete their intracellular granules, as measured by release of proteoglycans, without reorientation of the granules (630). This activation by anti-CD2 antibodies was induced when $F(ab')_2$ fragments were used, excluding the involvement of FcγR (630). However, when a single anti-CD2 antibody was used, signal transduction, as shown by increased $[Ca^{2+}]_i$ and cytotoxicity, was induced by interaction of the Fc portion of the anti-CD2 antibody with the CD16 FcR, since the $F(ab')_2$ fragment of the anti-CD2 antibody did not induce signal transduction and anti-CD16 antibodies partially blocked signal transduction induced by the intact anti-CD2 IgG antibody (631).

Monoclonal antibodies that block cytotoxicity mediated by NK cells from the catfish *Ictalurus panctatus* have been generated by immunization with enriched fish cytotoxic lymphocytes (271). These antibodies also react with human NK cells and inhibit the cytotoxicity of human NK cells and non-MHC-restricted CTLs against a variety of target cells (632, 633). The antibodies precipitate a heterodimer of 41 and 38 kDa from fish cells and of 43 and 38 kDa from human cells (271, 632, 633). These results, together with the studies (561) suggesting common target molecules on the fish parasite *Tetrahymena pyriformis* and NK cell-sensitive target cells, suggest that NK cells recognize target cells as using a receptor-ligand system that is highly conserved during evolution.

Preliminary reports have described other possible NK cell receptors. Ortaldo *et al.* (634) showed that antiidiotypic antibodies directed against an antibody specific for a glycoprotein of K562 cells react with an 80-kDa molecule on NK cells, block target binding and lysis by NK cells, and, when used to pretreat NK cells, enhance cytotoxicity and induce IFN production. Timonen *et al.* (635) have shown that certain antibodies to the $F(ab')_2$ of IgG specifically stain LGLs, precipitate predominantly a 60-kDa molecule from them, and block lysis by preventing postbinding reorientation of the effector cells. Interestingly, an antibody that reacts with the p48 lamininlike molecules on LGLs cross-reacts with the Ig light chain (636).

Of the different molecules for which a role has been proposed in NK-target cell binding or in postbinding events during NK cell-mediated cytotoxicity, only CD16 and CD2 have been shown to act directly in the signal transduction and activation of the cytotoxic mechanisms (630, 637). Only in the case of CD16 molecules has activation of NK cell cytotoxic and other functions been shown to be induced by their natural ligand, immune-complexed IgG (637). The large number of molecules that have been proposed to be NK cell receptors for target cells or to play some role in NK cell-mediated cytotoxicity is reminiscent of the confusion in the T cell receptor field before the TCR was identified. It is possible to speculate that most of the molecules described so far do not represent real receptors and that some of the results are artifacts due to a direct effect of the antibodies used on the NK cells, independent of any role of the recognized molecules in the cytotoxic process. However, unlike CTLs, NK cells do not appear to have antigenic specificity or clonally distributed receptors. Yet heterogeneity of NK cell and selectivity for target cells have been shown by competition experiments and by analysis of the selectivity of NK cell clones. Thus, the selectivity of NK cells might be determined by the relative expression of several cell surface molecules and receptors, and all of the molecules discussed above might play some role in either the recognition or the postbinding phases, with no single molecule playing a unique and essential role. Different sets of molecules might be involved in each combination of NK-target cells and a heterogeneity in the functional role of the various molecules might exist at both the NK and target cell levels.

C. Mechanisms of Cytotoxicity

The studies on the mechanism involved in T and NK cell-mediated cytotoxicity have been recently reviewed by Young and Cohn (638) and by Carpén and Säkselä (639). In this review, therefore, these studies are

briefly summarized, and only those aspects particularly relevant to the understanding of NK cell biology are emphasized.

Binding of NK cells to target cells occurs rapidly at both 4°C and 37°C (640, 641) and requires Mg^{2+} but not Ca^{2+} (641-644). After binding to the target cells, the NK cell undergoes a series of events known as activation and programming of the target cell to lysis. These events are temperature dependent (optimal temperature, 37°C), Ca^{2+} dependent, and sensitive to Ca^{2+} channel blockers and calmodulin inhibitors (406, 643-648). As previously discussed, initiation of NK cell activation might depend on an enhancement of phosphoinositide metabolism that is independent of extracellular Ca^{2+}, and it is triggered by interaction of CD16 FcγR with antibody-coated target cells or of other unknown NK cell membrane structures with NK cell-sensitive target cells. The formation of 1P3 and IP4 induces an increase in $[Ca^{2+}]_i$, first by release of Ca^{2+} from intracellular stores and then by the influx of extracellular Ca^{2+}, which is required for maintenance of increased levels. Chelation of extracellular Ca^{2+} probably prevents continuation of the lytic mechanism at this stage. An increase in cAMP has been shown to inhibit NK cell-mediated cytotoxicity; although inhibition of NK-target cell conjugates has been observed when cAMP levels in NK cells are increased (649), the major effect of increased cAMP is probably inhibition of the increased phosphoinositide metabolism (601, 604). Inhibition of NK cell activity by interaction with monomeric IgG is also primarily mediated by elevation of cAMP (650). Following the enhancement of phosphoinositide metabolism, activation of protein kinase C is probably involved in the cytotoxic mechanism, as indicated by inhibition of cytotoxicity by specific inhibitors (602).

The morphological analysis of NK-target cell interation showed broad cell to-cell adhesion of NK cells with the target cells, evidence of activation and degranulation in the NK cells with membrane material of probable granule origin present in the space between the two cells (284, 651). NK cells are often deeply invaginated and in some cases have been observed within the cytoplasm of the target cells, in a vesicle completely surrounded by a membrane and without communication with the extracellular medium. This phenomenon is defined as emperipolesis (284, 652).

Several observations indicate that the activation of lysis may require the release of fatty acids from the cell membrane. Exposure of PBLs to NK cell-sensitive target cells increases phospholipid methylation, and natural killing is reduced by the inhibition of methyltransferases as well as by inhibitors of phospholipase A_2 (576, 653), although some caution should be exerted in interpreting the specificity of phospholipase A_2

inhibitors (654). These findings suggest that metabolism of arachidonic acid is required for NK cell activity. However, inhibition of cyclooxygenase and prostaglandin synthesis does not affect NK cell activity, and various prostaglandins inhibit NK cell activity at binding and postbinding stages by increasing cAMP levels in the NK cells (655, 656). IFN and IL-2 activation of NK cells reduces the sensitivity to inhibition by prostaglandin and cAMP-elevating agents (655, 657). Inhibition of lipooxygenase decreases NK cell cytotoxicity, suggesting a role for endogenous lipooxygenase metabolites (leukotrienes) in NK cell activity (654, 658, 659). Recently, it has been shown that specific inhibitors of leukotriene C_4 (LTC_4) synthesis inhibit NK cell cytotoxicity and that addition of LTC_4 prevents the inhibition, suggesting an essential although unknown role of this arachidonic acid metabolite in cytotoxicity (659).

The lymphocyte-dependent events in NK cell-mediated cytotoxicity are followed by cytolysis of the target cells during the killer cell-independent lysis (KCIL) (642). This latter phase requires neither Ca^{2+} nor Mg^{2+}, but it is relatively sensitive to reduced temperature, prostaglandin E_2, heterologous anti-LGL antibody, and proteolytic enzymes (406, 642, 645). Recent studies have shown a rapid initial phase of Ca^{2+}-independent KCIL with a calculated half-life of less than 3 minutes (660). This rapid phase, which is independent of temperature over the range 10-37°C, is followed by a very slow, Ca^{2+}-independent disintegration of additional target cells (660). This second slow phase is temperature dependent and probably mediated by soluble factors released into the supernatant during effector-target cell interaction in the presence of Ca^{2+} (660).

Several morphological and metabolic inhibitor studies suggest that lysis is mediated by a vesicular secretory mechanism, involving polarization of granules to the part of the effector cell in contact with the target, followed by discharge of the granular content (see Ref. 639 for a complete review of directed exocytosis). Drugs that block vesicular secretion in other cell types inhibit NK cell killing without affecting the ability of the effector cells to bind the target cells (661). Degranulating agents both deplete the granules from LGLs and inhibit killing (643). The programming phase has been shown to involve transfer of a protease-sensitive material from the effector cells to the target cells (662, 663).

Wright and Bonavida (664-666) demonstrated that a soluble lytic factor is secreted by NK cells following lectin stimulation or NK-target cell interaction. This NK cell cytotoxic factor (NKCF) is lytic for NK cell-sensitive target cells, but not for most NK cell-resistant target cells (664, 665, 667). Detailed studies have shown that successful NK cell-mediated lysis requires that the target cells: (1) be recognized by NK cells,

allowing conjugate formation, (2) be able to induce release of NKCF (or other lytic mediators) from NK cells, and (3) be sensitive to the effect of the lytic mediators (573, 667). IFN-treated cells form conjugates with NK cells and are sensitive to NKCF but fail to induce release of the factor (573). These findings might explain the failure of IFN to protect target cells from ADCC mediated by NK cells (503): The interaction of the antibodies with FcγR on NK cells may induce release of lytic mediators, circumventing the step blocked by IFN treatment of the target cells.

NKCF is probably composed of more than one cytotoxic factor, and different factors can be active on different target cells. When the U937 cell line is used as a target for measuring NKCF activity, most of the activity is mediated by TNF, indicating that TNF is one component of NKCF and is produced by NK cells upon interaction with target cells (668, 669). When TNF-resistant cell lines such as K562 are used, the presence in NKCF of lytic factors different from TNF can be clearly demonstrated (669-672). However, these lytic molecules have not yet been purified, and molecular weights between 5,000 and 50,000 have been reported (668, 671, 672). A 50-kDa lytic molecule that cross-reacts with both TNF and lymphotoxin has been demonstrated in the granules and the cytoplasm of CTLs (673). Whether this lytic molecule is also present in NK cells and is related to NKCF remains to be determined. The characteristics of the lysis mediated by NKCF, especially the slow kinetics of lysis, make it unlikely that NKCF is the only mediator of lysis and participates in the rapid phase of disintegration of the target cells (660), although it is possible that faster kinetics of lysis is induced when high local concentrations of NKCF are reached in the contact area between effector and target cells. However, the slow second phase of target cell disintegration has been shown to be mediated by soluble factors produced by NK cells in the supernatant fluid and probably is induced by the lytic effect of NKCF (660).

Work from several laboratories has established that during NK cell-mediated lysis tubular lesions with an average internal diameter of 150-170 Å are observed on the target cell membrane and that isolated granules are able to mediate the formation of similar lesions (reviewed in Ref. 638). The granule molecule able to form the pores is a 70-kDa protein called pore-forming protein (PFP), or perforin (638). PFP requires Ca^{2+} for pore formation in membranes and it is rapidly aggregated and inactivated by the presence of Ca^{2+} in the medium; thus, PFP cannot represent a lytic factor present in the supernatant fluid, such as NKCF. Both human and murine perforins cross-react with antibodies to the C9 component of complement, another molecule able to polymerize to form pore structures in membranes in the presence of

Zn^{2+} (638). Human PFP was cloned and the high homology with C9 was confirmed by the nucleotide sequence (674). Whereas resting CTL precursor cells do not contain PFP and accumulate it only after stimulation, a low level of PFP can be isolated from the granules of human resting NK cells (675). NK cells are the only resting lymphocytes expressing detectable amounts of PFP constitutively (675).

It was initially reported that PFP isolated from freshly obtained human NK cells was able to form functional pores in liposome membranes but that it lacked efficient hemolytic activity, as measured on SRBCs (675, 676). It was also observed that NK cells are resistant to the lysis mediated by PFP, suggesting that they are protected from autolysis during cell-mediated cytotoxicity (677, 678). C9-mediated lysis is inefficient on homologous RBCs and nucleated target cells; therefore, lysis of autologous cells is probably prevented when complement is activated *in vivo* (678, 679). This homologous restriction is mediated by a 65-kDa protein called homologous restriction factor (HRF), related to C8 and C9 and present on RBCs (679) and nucleated cells (678, 679). By complexing rapidly with attacking C8 and C9 molecules, HRF is thought to interrupt the C polymerization process that leads to channel formation, and it has also been shown, in high concentrations, to prevent lysis of RBCs by the C9-related PFP and by NK cells in ADCC (679). It was postulated that the resistance of NK cells and CTLs to PFP was mediated by membrane and granule-associated soluble HRF (679, 680). However, human PFP from NK cells shows a species preference (i.e., it is unable to lyse RBCs from sheep and other species) but not an homologous restriction (i.e., it lyses human and mouse RBCs) (674, 678). These data explain the previously described lack of hemolytic activity of human PFP when tested on SRBCs, and exclude that the resistance of NK cells to PFP is mediated by HRF (674, 678). NK cells and CTLs, like other nucleated targets, are resistant to lysis by homologous but not heterologous complement; however, these cell types are resistant to both homologous and heterologous PFP (678). The resistance of NK cells to PFP is a property of resting NK cells and it is increased by stimulation with IL-2 (678). The mechanism of protection of cytotoxic cells against PFP is unknown and could be mediated by a protein with similar functional characteristics but distinct from HRF.

The rapid phase of target cell lysis mediated by NK cells is consistent with the type of cytotoxic mechanism mediated by PFP, and PFP is always present in NK cells, both freshly obtained from peripheral blood or activated. However, PFP-mediated lysis is not a universal mechanism of cell-mediated lysis, and much controversy exists about whether it is the major mechanism of lysis, mediated by CTLs (681). *In vitro* grown,

IL-2-dependent CTLs have LGL morphology and contain PFP in their granules. However, highly efficient CTLs freshly obtained from allo-immunized animals do not have LGL morphology, do not contain detectable PFP, and no channels are demonstrable on the lysed target cells (681). Also, CTLs can kill certain target cells in the absence of Ca^{2+} in the medium, contradicting the granule exocytosis model (682). Thus, these CTLs probably use a different mechanism of lysis, and it has been proposed that direct interaction between CTL receptors and target cell antigens may irreversibly damage target cell membranes, activating an endogenous mechanism of cell lysis (681). There is indeed a major difference in the KCIL following NK cell or CTL interaction which has received little attention but may reflect fundamentally different mechanisms of lysis: If Ca^{2+} is chelated during lysis mediated by non-cultured CTLs, the release of ^{51}Cr continues for 1–2 hours, showing a slow lysis of the programmed target cells (683), whereas in the case of ADCC (684) or spontaneous lysis mediated by NK cells (73) an almost immediate arrest of ^{51}Cr release is observed upon chelation of Ca^{2+}.

The mechanism of cell-mediated lysis following channel insertion in the membrane is referred to as colloid osmosis (685). The lethal hit initiates with a progressive series of cytoplasmic convulsive movements in the target cells accompanied by nuclear and plasma membrane blebbing, termed zeiosis, which precedes an increase of transmembrane fluxes and loss of cytoplasmic contents (685).

Russell (686) has proposed an alternative model of "internal disintegration" to explain the mechanism of cell-mediated lysis, according to which lymphocytes trigger an autocatalytic cascade within the target, which results in nuclear membrane damage and DNA fragmentation. DNA fragmentation has also been demonstrated during NK cell-mediated lysis, although the kinetics is slower than that observed with CTLs, suggesting that in addition to cell-to-cell contact, a soluble factor such as NKCF is involved in inducing the intracellular damage (687). Human target cells present little or no DNA degradation when lysed by either human or murine cytotoxic cells (688, 689); because the difference in DNA degradation depends on the species of the target cells, not the effector cells, the degradation is probably due to activation of target cell endogenous endonucleases.

Proteases have been implicated in the mechanism of cell-mediated lysis. ADCC (690) and spontaneous cytotoxicity (691, 692) mediated by NK cells have been shown to be inhibited by various synthetic and naturally occurring protease inhibitors, especially chymotrypsin-specific inhibitors. Protease inhibitors blocked NK cell-mediated lysis at or after the postbinding Ca^{2+}-requiring step (690). However, these original

observations were difficult to interpret because the studies were performed with intact cells, and the most effective protease inhibitors used, the single-amino acid chloromethylketones, also induced nonspecific alkylation.

Human NK cells contain a urokinase-type plasminogen activator in vesicles that polarize during conjugate formation with target cells (693). Serine esterases 1 and 2 (or granzymes A and B), with no plasminogen activator activity, have been identified in murine CTLs (694); equivalent esterases have been cloned from human cells, and their mRNAs have been demonstrated on fresh peripheral blood NK cells (695-697). Serine esterases are secreted from lymphocytes stimulated by calcium ionophores or by interaction with target cells and might be involved in cytotoxicity (638, 694, 697). Using various protease inhibitors and measuring the ability to degrade serum amyloid A, Zucker-Franklin *et al.* (698) showed that NK cells, but not other PBLs, carry several enzymes with different substrate specificities, some of which may be involved in cytotoxicity.

Hudig *et al.* (699) and Zunino *et al.* (700) analyzed the requirement for proteases in the lysis mediated by granules obtained from RNK-16 cytotoxic rat lymphocytes. The chloromethylketone Z-Gly-Leu-Phe-CH_2Cl and the irreversible mechanism-based inhibitors 7-amino-4-chloro-3-(2-phenylethoxy)-isocoumarin and dichloroisocoumarin completely blocked RNK-16 granule-mediated cytolysis, demonstrating a requirement for trypsin- and chymotrypsinlike proteases in the lysis mediated by the granules. Although the mechanism of action of the proteases is unknown, it is possible that lytic molecules, e.g., PFP, are present in the granules in an inactive form and that proteolysis is required for activation. A potential substrate could be an inhibitory PFP-binding protein similar to HRF (680).

The granules of NK cells contain proteoglycans of the chondroitin sulfate A type which are released during cytotoxicity or activation by anti-CD2 antibodies (701-703). A role for proteoglycans in the mechanism of cytotoxicity or in the protection of effector cells has been proposed (701-703), although it was shown that a significant decrease of proteoglycan synthesis induced by culturing NK cells in β-D-xyloside neither decreased NK cell cytotoxic activity nor increased autolysis (704).

D. Regulation of NK Cell Cytotoxic Activity and Proliferation

Infection of mice with viruses, certain microorganisms, and their products has been shown to result in enhanced NK cell cytotoxicity (17, 705), and IFN, a potent NK cell activator, was found to be produced under most of the *in vivo* or *in vitro* conditions in which augmentation of NK cell activity has been observed (34, 80, 104, 591, 706).

IFN efficiently enhances the cytotoxic activity of NK cells (34, 591). This effect can be readily demonstrated and quantitated by preincubating lymphocytes in the presence of IFN and then testing their cytotoxic ability against target cells unable to induce IFN production (34). All three known types of IFN, fibroblast (β), different species of leukocyte type I (α), and leukocyte type II or immune (γ) are able to enhance human NK cell cytotoxicity (272, 408, 707). However, IFN-γ is not effective with cells from all donors and always enhances NK cell cytotoxicity at a lower extent and with a slower kinetics than does IFN-α or IFN-β (708–711). Human NK cells, as well as other lymphocytes, express high affinity receptors for IFN-α/β and IFN-γ (712). IFN treatment of NK cells induces 2′,5′-oligoadenylate (2′,5′A) synthetase and, under appropriate experimental conditions, 2′,5′A augments NK cell cytotoxicity, suggesting that, as in the case of IFN antiviral activity, the pathway of IFN-mediated augmentation of NK cell cytotoxicity may involve 2′,5′A (713, 714). However, although most species of recombinant IFN-α enhance NK cell cytotoxicity, recombinant IFN-α J, with potent antiviral and antiproliferative activity, fails to do so (715). IFN-α J binds to the same receptors as the other IFN-α and blocks the NK cell-activating effect of the other species of IFN-α (716). These results indicate possible differences in the mechanisms of action of IFN in inducing antiviral activity or augmenting NK cell cytotoxicity.

In addition to IFN and IFN-inducing cells (such as virus- or mycoplasma-infected cells), other IFN inducers, such as viruses and polyinosinic-polycytidylic acid (poly I:C), also enhance NK cell activity by inducing IFN production by cells present in the cell preparations used as a source of NK cells (34, 591). The IFN-dependent enhancement of the cytotoxic activity of NK cells is very rapid and requires *de novo* protein synthesis but not cell proliferation (34). Although NK cells show increased cytotoxic activity after IFN treatment, they do not show a pattern of target cell specificity different from that of untreated ones. However, they can very efficiently kill target cells that are not very sensitive to the killing by untreated NK cells (34). The increase in killing ability as a result of IFN stimulation is proportionally greater against these less susceptible target cells than against very susceptible target cells; the number of cells lysed increases up to 20-fold in the former situation, whereas that of cells lysed when the NK-susceptible cell line K562 is used, for example, increases only 1.5- to twofold (591). IFN-treated NK cells are also able to lyse fresh tumor target cells, which are relatively resistant to lysis by nonstimulated NK cells (717). IFNs stimulate the cytotoxic activity of NK cells only and do not endow T or B cells with non-MHC-restricted cytotoxic activity (95, 272).

IFNs have been shown to affect NK cell cytotoxicity through at least three different mechanisms: (1) by increasing both the number of NK cells able to bind to their targets and the proportion of cytotoxic cells within the NK cell population (59, 95, 502, 718-721), (2) by accelerating the kinetics of lysis (720, 721), and (3) by increasing the recycling ability of active NK cells (76, 502).

The major changes in NK cell morphology after IFN treatment are observed in the structure of the granules. Cytoplasmic granules containing an electron-dense matrix or PTA become virtually undetectable and are replaced by large vesicular structures with, often, a residual electron-dense matrix surrounded by aggregates of round vesicles or membranous myelin figures (281).

The effect of IFN on the ability of NK cells to mediate ADCC is more controversial (34, 591, 722-725). In many of the reports that claim enhanced NK cell ADCC activity, the researchers have disregarded the confounding effect of (1) increased spontaneous (antibody-independent) background killing of the target cells mediated by NK cells and (2) the possibility that the high concentrations of antibodies used to sensitize the target cells trigger both monocyte and NK cell cytotoxicity. However, experimental conditions have been reported that rigorously demonstrated an enhancing effect, although modest, of IFN on NK cell ADCC activity (724). Spontaneous cytotoxicity and ADCC are two functions mediated by the same NK cell type. These functions may depend on discrete mechanisms of target cell recognition that activate the same or two different lytic processes. Although the differential effect of IFN on spontaneous cytotoxicity and ADCC might be considered evidence for separate mechanisms, it is more likely that the enhancing effect of IFN on ADCC activity of NK cells is difficult to demonstrate because the interaction of IgG on target cells with CD16 FcR on NK cells determines optimal stimulation of NK cells and maximal killing that cannot be further increased by IFN. The inefficiency of IFN in enhancing ADCC activity is therefore analogous to its inefficiency in enhancing the lysis of target cells very sensitive to NK cell-mediated lysis, as discussed above. In support of this interpretation, it was reported that the enhancing effect of IFN on ADCC activity of NK cells is observed only when suboptimal concentrations of antibodies are used (726, 727).

In vivo IFN treatment of patients determined in most cases an increase in NK cell activity that is, however, often transient and in some cases followed by depression (728-733). IFNs *in vitro* do not induce NK cell proliferation, but *in vivo*, in the mouse, IFNs were shown to induce blast formation, DNA synthesis, and probably proliferation of NK cells (734).

Suppressor cells have been described by several investigators in the murine system and appear to be responsible for the depressed NK cell activity observed in animals treated with IFN (735) or with carrageenan (736). Murine suppressor cells for NK cell activity have been shown to be both macrophages (735-740) and T cells (741, 742). Prostaglandins (PGs), which inhibit spontaneous cytotoxicity *in vitro*, are probably the soluble mediators of the suppression mediated by macrophages (743). In humans normal granulocytes (104) and, to a lesser extent, peripheral blood monocytes (104, 744, 745) have suppressor activity on NK cells. Tumor-associated lymphocytes and macrophages from patients with different types of malignancies have been found to inhibit NK cell cytotoxicity (746-750). Indomethacin, in some cases, reverses the inhibition, suggesting that PGs are also involved in the suppressive effect mediated by human macrophages (748, 751). PGs of the E series suppress human NK cell activity (751-754); however, IFN treatment of NK cells decreases their sensitivity to this suppressive effect (755). Activation of human lymphocytes in culture induces the generation of suppressor cells for NK cell activity (756, 757). In one study these suppressor cells were identified as HNK-1$^+$, FcμR$^+$ but FcγR$^-$, CD16$^-$ non-T cells (756). On the basis of this phenotype, it was suggested that NK cells themselves can function as immunoregulators, controlling their own cytotoxic activity. Suppressor cells for NK cell activity have also been found in normal human cord blood (341); these suppressor cells are probably in part responsible for the reduced NK cell activity mediated by human cord blood lymphocytes, notwithstanding a normal proportion of both LGLs (341) and CD16$^+$ cells (95). The cord blood suppressor cells have been identified as medium-sized CD3$^+$, FcγR$^+$ T cells. IFN treatment of these cells abolishes their suppressive activity (341).

TGF-β and platelet-derived growth factor have inhibitory effects on NK cell-mediated cytotoxicity (758, 759). TGF-β prevents the enhancement of NK cell cytotoxicity induced by IFN, but not that induced by IL-2 (758).

T cell growth factor or IL-2 is a potent enhancer of NK cell activity *in vitro* and *in vivo* (708, 760-764). The optimal doses of IL-2 able to enhance NK cell-mediated cytotoxicity are 100- to 1000-fold higher than those required for maintaining proliferation of activated T lymphocytes, and antibodies against the p55 chain (TAC or CD25 antigen) of the IL-2 receptor are unable to prevent NK cell enhancement of cell cytotoxicity (708, 763). These data suggested the existence on NK cells of an IL-2 receptor different from the high-affinity IL-2 receptor associated with the TAC antigen and became interpretable when it was discovered that

the high-affinity IL-2 receptor is composed of the p55 chain (TAC antigen) and a second p70 chain (765). Resting NK cells express higher levels of the p70 chain than do other lymphocytes (766). The p70 chain, when not associated with the p55 chain, binds IL-2 with an affinity approximately one hundredth that of the complete receptor and is responsible for the response of resting NK cells to IL-2 (766, 767). The same high concentrations of IL-2 induce a modest production of IFN-γ from resting NK cells (708). It was originally reported that the enhancement of NK cell cytotoxicity by IL-2 was mediated by endogenously produced IFN-γ (761, 763). However, the use of impure anti-IFN-γ antibody preparations was shown to be responsible for some of the results originally reported (768), and there is now general agreement that the effect of IL-2 is direct and not mediated by IFN-γ, because (1) anti-IFN-γ monoclonal antibodies do not prevent the enhancement of NK cell cytotoxicity mediated by IL-2 (708, 768-770), (2) the enhancement of cytotoxicity mediated by IL-2 precedes by several hours the appearance of detectable IFN-γ in the supernatant fluids (708, 770), and (3) the production of IFN-γ by NK cells requires the participation of class II MHC-positive accessory cells, whereas the enhancement of cytotoxicity is independent of accessory cells. The morphological aspect of IL-2-activated NK cells is different from that of IFN-activated NK cells: The morphology of the IL-2-activated cells is altered, with expansion of the Golgi apparatus and increase in the number of electron-dense granules and vesicles; the granules, however, do not show the deaggregation of the electron-dense matrix observed in IFN-treated NK cells (281). IFN and IL-2 synergize in their enhancing effect on NK cell cytotoxicity (770, 771). Short-term treatment with IL-2 (up to 24 hours) enhances the cytotoxicity of purified $CD3^-$, $CD16^+$ NK cells and does not endow freshly obtained $CD3^+$ cells with non-MHC-restricted cytotoxicity when tested against NK cell-sensitive or -resistant target cells (156, 708).

The enhancement of cytotoxic activity of NK cells is demonstrable after 3-6 hours of incubation and does not require proliferation (708). Incubation of PBLs with IL-2 in the absence of other stimuli, however, induces moderate cellular proliferation after 3-4 days of incubation (708). Analysis of the proliferating cells by autoradiography after treatment of PBLs with IL-2 has shown that a proportion of both NK and T cells is induced into the cell cycle (708). However, limiting dilution experiments (93, 772) and colchicine blockage experiments (93) showed that the majority of mature peripheral blood NK cells can be induced into the cell cycle by IL-2 alone, whereas only a minor proportion of low-density T cells is induced to proliferate. The effect of endogenously produced IFN-γ on the IL-2-induced proliferation of NK cells is controversial,

but inhibition of proliferation by anti-IFN-γ antibodies was shown in cultures of both human (773) and murine (774) NK cells. Induction of proliferation of NK cells requires the same high concentrations of IL-2 as the enhancement of cytotoxic activity or production of IFN-γ and does not depend on expression of the TAC antigen on NK cells (93). It is therefore likely that induction of proliferation is also mediated through the p70 chain of IL-2 receptor, with intermediate affinity for IL-2. However, IL-2 induces expression of TAC (CD25) antigen on purified NK cells after 2–4 days of culture (191) and anti-TAC antibodies suppress proliferation (93), suggesting that expression of the high-affinity IL-2 receptor is required for maintenance of proliferation. In addition to TAC (CD25) antigen, other activation antigens such as TfR, CD38, and class II MHC antigens become strongly expressed on proliferating NK cells (191). When NK cells revert to a resting state, cell surface expression of CD25, CD38, and TfR decreases or ceases, whereas NK cells remain class II MHC positive (100). Recently, cell surface expression Leu-23, an antigen present as a 60-kDa heterodimer composed of two chains of 32 and 28 kDa, has been shown to be induced and phosphorylated on the large majority of NK cells after a few hours of stimulation with IL-2 (775), confirming that the majority of resting NK cells respond to IL-2.

Culture of PBLs with IL-2 for a few days induces the generation of non-MHC-restricted cytotoxic cells, termed LAK cells, that are able to efficiently lyse NK cell-resistant target cells, including fresh tumor cells (776–778). Infusion of autologous *in vitro* generated LAK cells in patients, together with recombinant IL-2, has resulted in at least partial regression of solid tumors in a low but significant proportion of patients (27). Although LAK cells have been originally described as $CD3^+$ cytotoxic cells originated from $CD3^-$ precursors (777), studies from many groups have clearly shown that most of the cytotoxicity mediated by LAK cells is due to IL-2-activated $CD3^-$ NK cells, although a minor component could be due to non-MHC-restricted cytotoxic $CD3^+$ T cells (156, 708, 764, 779–781). The cytotoxic cells present in the peripheral blood of patients receiving IL-2 have also been shown to be mostly or exclusively $CD3^-$ NK cells (764). The identification of NK cells as the major mediators of LAK cell cytotoxic activity, as measured by *in vitro* assays, does not, however, provide information on the cell type responsible for tumor regression *in vivo* in patients treated with unfractionated, IL-2-treated PBLs, composed, in large proportion, of T cells. The definition of LAK cells does not identify a single or novel cell type, but rather identifies a phenomenon, i.e., the ability of IL-2 to enhance the cytotoxicity of NK cells and to endow certain T cells with non-MHC-restricted

cytotoxic ability. The analysis of the LAK cell phenomenon has generated little original information on the biology of NK cells, and its description is beyond the scope of the present review.

The LAK cell phenomenon appears in part to be similar to the generation of "anomalous" killer cells in mixed-lymphocyte cultures (782–786), although it remains unclear, in this latter system, whether the progenitor cells are NK cells, T cells, or both (784, 785).

NK cells, but not T cells, have been shown to be capable of chemokinesis and chemotaxis when exposed to C5a, *N*-formyl-methionyl-leucyl-phenylalanine, and casein (787, 788), suggesting that NK cells have receptors for these typical stimulants of PMNs and monocytes. IFN and IL-2 increase the locomotor ability of NK cells, without increasing their ability to respond to chemoattractants (789). The migration of NK cells can be demonstrated by using nitrocellulose filters, but not polycarbonate filters, which require the migrating cells to behave as adherent cells (790). Treatment of NK cells with phorbol diesters, however, activates NK cells, enhancing their cytotoxic ability (133, 791) and making them able to adhere to various substrates (792). Phorbol diester-activated NK cells migrate through polycarbonate filters as adherent cells (791). These data, together with the ability of IL-2 to induce adherence of NK cells, but not T cells, to endothelial cells (793, 794), suggest that the change of adherence capability and migratory behavior of NK cells following activation may be determinant to induce activated NK cells to adhere to vascular lining and localize in tissues.

The ability of IL-2-activated NK cells to adhere to plastic has been utilized for obtaining enriched preparations of activated NK cells in both humans and experimental animals (795, 796). PBLs or spleen cells are cultured for 24 hours in the presence of high doses of IL-2, then the nonadherent cells are further cultured in the presence of IL-2; after 14 days of culture a several hundredfold proliferation of the adherent cells is observed and the majority of the collected cells have the phenotype of NK cells with potent cytotoxic activity (795, 796). This method has been proposed as a relatively simple technique to obtain activated NK effector cells for antitumor adoptive immunotherapy (796).

Irradiated B lymphoblastoid cell lines, in the presence of a source of IL-2, augment proliferation of mature NK cells, enhance NK cell clonal efficiency, and facilitate the growth of IL-2-dependent NK cell clones (93, 99, 191, 797). Although the exact mechanism of action of the cell line is unknown, it has been shown that they do not increase the frequency of NK cells entering the cell cycle in response to IL-2, but rather facilitate the continuous proliferation of the NK cells (93). During culture of total PBLs with irradiated B lymphoblastoid cell lines,

cell surface activation antigens are rapidly induced (100, 192). NK target cell structures present on the cell lines used as a stimulator may play a direct role in inducing NK cell activation because the MHC-negative, NK-sensitive K562 cell line has been reported to induce proliferation of NK cells but not T cells (798).

Certain irradiated B lymphoblastoid cell lines, such as Daudi and RPMI 8866 cells, but not various T or myeloid cell lines induce preferential proliferation of $CD16^+$, $NKH\text{-}1^+$, $CD3^-$ human NK cells when cocultured with total PBLs (100). After 10 days of cultures, an average fourfold increase in total cell number is observed, with NK cells representing between 50 and 90% of the total cells recovered (100). NK cells can be easily purified from these cultures. This represents a technically simple method for obtaining large quantities of pure NK cells without the need of adding IL-2 to the culture and has been instrumental in obtaining large numbers of human NK cells for molecular and biochemical studies (78, 637, 675, 799). The preferential NK cell proliferation is not observed when PBLs are stimulated by irradiated allogeneic PBLs in a classical mixed-lymphocyte culture or when PBLs are cultured in the pressnce of IL-2 alone (100). NK cell proliferation occurs in the absence of exogenously added IL-2, but is blocked by anti-IL-2 antibodies and requires the presence of $CD4^+$ T cells in the starting PBL preparation, suggesting that the $CD4^+$ T cells stimulated by the allogeneic lymphoblastoid cell lines produce IL-2 that, together with irradiated cell line, induce the preferential proliferation of NK cells (100). An alternative, or additional, interpretation may be suggested by the observation (492, 493) that mitomycin C-treated autologous T cell blasts are able to induce generation of NK-like cells from $CD3^-$ small-lymphocyte precursors. It is possible that $CD4^+$ blasts, generated by allogeneic stimulation with the irradiated B cell lines, similarly induce proliferation/differentiation of NK cells and/or NK precursor cells.

Mitogenic lectins are unable to induce proliferation of purified human NK cells (191), although they can enhance phosphoinositide turnover and increase $[Ca^{2+}]_i$ in both NK and T cells (M. Cassatella, personal communication). The inefficiency of NK cells to produce growth factor such as IL-2 might be responsible for the failure of lectins to induce NK cell proliferation. Phorbol diesters and calcium ionophores together have been shown to induce proliferation of Percoll-enriched preparations of NK cells (800); however, using the same stimuli, we (L. London and G. Trinchieri, unpublished observations) have been unable to obtain proliferation of NK cells purified by positive selection of $CD16^+$ cells, raising the possibility that contaminant accessory or suppressor cells may regulate NK cell proliferation in these experimental conditions.

The effect of IL-4 on NK cell cytotoxicity and proliferation is controversial, with opposite results reported with human and murine NK cells. IL-4 has no effect on the cytotoxic ability of human resting NK cells, but it inhibits in a dose-dependent manner the IL-2-induced cytolytic activation of NK cells, but not the IFN-induced activation (801, 802). IL-4 acts directly on purified NK cells and does not require accessory cells (802). In the murine system, however, IL-4 alone was shown to induce non-MHC-restricted cytotoxic cells against fresh tumor cells and to augment the effect of IL-2 on the generation of cytotoxic cells (803, 804), although, unlike IL-2-induced cytotoxic cells, T cells, not NK cells, represent the major component of the IL-4-induced cytotoxic cells (804). Indeed, IL-4 was shown to have a modest effect, if any, on the cytotoxicity and proliferation of purified murine NK cells or on spleen cells from *scid* mice, lacking T cells (229).

The possible effect of IL-1 on NK cell cytotoxicity and activation has not been extensively studied. IL-1 does not affect directly the cytotoxicity of NK cells, but might act synergistically with IL-2 or IFN in enhancing cytotoxicity of NK cells against certain tumor cells (805). This effect of IL-1 is possibly dependent on the ability of IL-1 to induce CD25 (p55 chain of IL-2 receptor) antigen on a proportion of human NK cells (806). The cytotoxicity of human NK cells is enhanced by treatment with high doses of TNF (807). TNF also acts synergistically with IL-2 in enhancing the cytotoxicity of human NK cells (807) and inducing generation of non-MHC-restricted cytotoxic cells (808).

E. Production of Lymphokines by NK Cells

NK cells have been described to be able to produce a large number of lymphokines. However, in many early studies contaminant cell types were present in the enriched NK cell preparation, and definitive identification of NK cells as the lymphokine producer cells was not provided.

Various factors and other substances might be preformed in the granules of NK cells and be secreted during interaction of NK cells with target cells or immune complexes. NKCF, PFP, esterases, proteoglycans, and various enzymes are included in this group of substances. A series of three probably distinct factors with activity on macrophages and other cell types have been found to be associated with cytoplasmic granules, obtained from both human NK cells and rat LGL leukemia RNK cells, and are released upon interaction with target cells or treatment with substances inducing degranulation such as Sr^{2+} (809–811).

Activation of intracellular microbicidal activity in rat and human alveolar macrophages was shown to be mediated by one of these

preformed NK cell cytokines, a protein of 10-20 kDa, heat and pH labile (809). Another NK granule-associated factor, released during granule secretion, was a leukocyte chemotactic factor inducing chemokinesis and chemotaxis of LGLs, neutrophils, and macrophages (810). A third factor, NK cell granule-macrophage activating factor, is a small protein (less than 10 kDa), heat stable and able to activate the tumoridical activity of bone marrow-derived macrophages in the presence of lipopolysaccharide (811). The optimal release of these factors from the granules requires ionic solubilization in 2 *M* NaCl, suggesting that they are tightly bound to an internal granule matrix. Because these factors are released in active form during degranulation, physiological mechanisms equivalent to this ionic solubilization should take place, and granule proteases may act to digest an internal matrix to liberate some molecules stored in an inactive form (810, 811). The granule-associated factors with activity on phagocytic cells might play role in the effect of NK cells *in vivo* against bacterial infections, as discussed in Section IX.

Factor(s) present in the supernatant fluid of NK cells activated by interaction with target cells stimulate a strong luminol-dependent chemiluminescence (CL) response in monocytes (812). This activation of CL in monocytes mediated by NK cells was found to be responsible for the CL response attributed directly to NK cells in previous studies (813, 814). The CL response described in NK cells after interaction with target cells was shown to be due to the presence of few contaminant monocytes stimulated by NK cells or NK products (812). NK cells are incapable of oxidative burst and do not produce superoxide anion during interaction with target cells (812, 815-817). NK cell cytotoxic activity does not require oxygen-dependent mechanisms, as shown by intact NK cell cytotoxic activity in chronic granulomatous disease patients (818). Because several hydroxyl radical (OH) scavengers inhibit cytotoxicity, it was proposed that OH is critical for NK cell cytotoxicity (815, 819, 2186). Because NK cells do not have NADPH oxidase acrivity, as was also confirmed by the impossibility to demonstrate in purified NK cells mRNA for the heavy chain of cytochrome b_{245}, an integral part of the NADPH oxidase system (M. Cassatella and G. Trinchieri, unpublished observations), it was hypothesized that OH scavengers are formed by the lipooxygenase pathway of arachidonic acid metabolism (819). However, the observations that OH scavengers inhibit NK cell cytotoxicity only when used at concentrations higher than those required to inhibit CL in monocytes (816) and that electron spin resonance spectroscopy does not reveal OH radical production in activated NK cells (820) strongly argue against the hypothesis that OH radical production plays a role in the early event of NK cell activation.

The possibility that NK cells might be able to secrete factors with NK-enhancing activity, such as IFN and IL-2, and thus be capable of self-regulation has generated much interest (34, 79, 821). In the early studies of NK cell cytotoxicity against cell lines able to induce IFN-α production, e.g., virus-infected target cells, it was not possible with the reagents available to unambiguously distinguish between NK cells and IFN-α-producing cells, although the fact that IFN-α-producing cells were all E-rosette negative—whereas about 50% of NK cell cytotoxic activity was recovered in the E-rosette-positive cell fraction—excluded a complete identity between the two cell types (34, 569, 706, 822). Several reports have, however, subsequently appeared, suggesting that the major IFN-α producer cells in peripheral blood were NK cells, based on results showing that the cells were found in the light-density fractions of a Percoll gradient and that they adhered to NK target cells (821, 823-826). Recently, however, several groups have shown that the IFN-α-producing cells in response to viruses, virus-infected cells, and other stimuli are HLA-DR$^+$ nonadherent cells, distinct from monocytes, dendritic cells, or T, B, or NK cells (92, 510, 827-829). Resting NK cells have null or very low ability to produce IFN-α (92, 510). The role of this IFN-α-producing cell type in the cytotoxicity of NK cells against virus-infected target cells will be discussed in Section IX.

NK cells are powerful producers of IFN-γ when stimulated with IL-2 (78, 708, 763, 830). The IFN-γ induced in total PBL preparations by IL-2 treatment is produced predominantly by NK cells and in part by T cells (708, 830). The production of IFN-γ by resting NK cells, as well as by resting T cells, requires, however, the participation of HLA-DR$^+$ accessory cells, with a mechanism still unclear (831). Because the majority of NK calls are rapidly induced by IL-2 to produce IFN-γ, it is likely that *in vivo* during an immune response the few antigen-specific T cells that may respond to antigen with production of IL-2 secondarily recruit NK cells as the major producers of IFN-γ; the role of NK cell-produced IFN-γ in B cell response is discussed in Section X.

The ability of NK cells to produce IL-2 is controversial. Although NK cell preparations have been reported to produce IL-2 (824, 832), the phenotype of the IL-2-producing cells was ambiguous and never corresponded to that of the majority of NK cells (824). IL-2 production has never been conclusively demonstrated using highly purified preparations of NK cells and the bulk of evidence, showing that NK cells cannot be induced to proliferate by a variety of mitogenic stimuli in the absence of IL-2-producing cells or an exogenous source of IL-2, suggests that NK cells are unable to produce IL-2 or are very poor producers. A comparison of T and NK cell clones, showing non-MHC-restricted cytotoxic

activity, showed that the majority of the T cell clones produced high levels of IL-2, whereas only two of 11 NK cell clones produced small levels of IL-2 (833).

NK cells have been shown to produce B cell growth factors (834, 835) and various types of colony-stimulating factors, as discussed in detail in Section VIII. During the study of the effect of human NK cells on bone marrow colony formation, it was found that NK cells, when cultured with bone marrow cells or NK-sensitive target cells, release low levels of TNF (668). This result was surprising because TNF was considered a macrophage product, but production of TNF by both NK and T lymphocytes was subsequently confirmed at both the protein (78, 260, 836, 837) and molecular (78, 260) levels.

Recently, study of the ability of human NK cells to produce various lymphokines was facilitated by the ability to obtain large numbers of highly purified NK cells from the cultures of PBLs and irradiated lymphoblastoid cell lines (100) and by the specific stimulation of NK cells through CD16 FcR ligands, i.e., Sepharose linked anti-CD16 antibodies or immune complexes (IgG antibody-coated RBCs or target cells) (78). Cross-linking of CD16 FcR or IL-2 treatment of highly purified NK cells induces low levels of IFN-γ and TNF production; the two stimuli, however, strongly synergize and high levels of both cytokines are released when NK cells are stimulated by the two stimuli together (78). Both stimuli induce transcription of the lymphokine genes and accumulation of mRNA transcripts in the cytoplasm; however, the synergistic effect of the two stimuli is observed at the mRNA accumulation level but not at the transcription level, suggesting that both stimuli induce lymphokine expression by acting at the transcriptional level, but that the synergistic effect is mostly posttranscriptional (78). The induction of transcription of lymphokine genes by CD16 ligands or IL-2 takes place in less than 20 minutes and mRNA accumulation does not require protein synthesis, suggesting a direct effect without other *de novo* produced proteins acting as intermediate messengers (78). CD16 ligands but not IL-2 induce phosphoinositide turnover and an increase of $[Ca^{2+}]_i$, originated from intracellular stores and from extracellular Ca^{2+} (637). The accumulation of mRNA and the induction of transcription by CD16 ligands but not by IL-2 require extracellular Ca^{2+}, indicating the importance of the increased $[Ca^{2+}]_i$ in the induction of transcription by CD16 ligands and the different signal transduction mechanisms used by the two stimuli (637).

Stimulation of purified NK cells with CD16 ligands and IL-2 induces high levels of mRNA accumulation and release of IFN-γ, TNF, GM-CSF, and CSF-1 (78, 799). Nonspecific stimulation with phorbol diesters and

calcium ionophore induces IFN-γ, TNF, GM-CSF, and IL-3 (799). In neither case was accumulation of transcripts for G-CSF, IL-1α, or IL-1β observed (799). The lack of detection of IL-1α or β mRNA was surprising, because previous studies have shown that NK cells are powerful producers of IL-1 in response to endotoxin (838, 839). However, NK cells, unlike monocyte/macrophages, are not stimulated to produce TNF by endotoxin (799), and it is possible that the IL-1 production in the NK cell preparation previously reported was due to contamination with a small number of monocytes, activated by NK cells as shown for the CL response, or that the IL-1 activity reported was due to a cytokine different from IL-1α or IL-1β.

VI. Interaction between NK Cells and the Central Nervous System

Bidirectional communication between the immune and central nervous systems provides the opportunity for coordinate mobilization of the specialized capacities of each system to sense and respond to environmental and autologous challenges (804). The study of neuroimmunology has been focused mostly on the neuroanatomy of lymphoid organs and shared or interdependent biochemical, functional, developmental characteristics of the two systems, with only limited emphasis on the effect of behavior on neuroimmunological communication (840). The NK cell system has been shown in many studies to be profoundly affected by neuroimmunological interactions, although the mechanisms of these interactions and their physiological significance are still unclear.

Several antigens present on NK cells are also expressed on cells of the central nervous system. The distributions of the HNK-1/Leu-7 and NKH-1/Leu-19 antigens in nervous tissues have already been discussed. In addition, the Thy-1 antigen is present on both murine T and NK cells and neurons.

The control of NK cell activity by the central nervous system is suggested by a decrease in NK cell activity *in vivo* following electrolytic lesions of the hypothalamus. Lesions in the anterior hypothalamus in the rat (841) and the median region of the hypothalamus in the mouse (842) were effective in inducing a decrease in NK cell activity lasting 1–2 weeks.

A series of clinical and experimental observations associates behavioral depression and stress with suppression of NK cell activity. A clinical syndrome characterized by general symptoms of remittent fever and persistent uncomfortable fatigue, often with severe depression, has been significantly associated with decreased NK cell activity in peripheral blood (843, 844). This syndrome has been called chronic fatigue

syndrome, low NK cell syndrome, or chronic active EBV infection, although many patients have a negative or normal anti-EBV titer. In many patients the $CD16^+$, $CD3^-$, NKH-1/Leu-19^+ NK cell subset is significantly reduced, whereas the $CD3^+$, NKH-1/Leu-19^+ one is present in normal proportions ($\sim 3\%$) and is responsible for most of the low NK cell cytotoxic activity mediated by the PBLs of these patients (844).

NK cell cytotoxicity was found to be significantly lower in a group of hospitalized depressed men than in matched controls (845). In patients with breast cancer, the level of NK cell activity was associated with various pathological parameters, such as nodal status; however, more than half of the baseline NK cell activity variance could be accounted for by factors such as patient adjustment, lack of social support, and fatigue/depression symptoms (846). NK cell activity is reduced in women undergoing conjugal bereavement (847, 848). Bereaved women showed reduced NK cell activity and increased plasma cortisol levels as compared to controls; however, anticipatory bereaved women also showed significantly reduced NK cell activity, although levels of plasma cortisol were comparable to those of controls; thus, the reduction of NK cell activity could not be explained on the basis of increased cortisol secretion (848). The importance of depression associated with commonplace stressful events was shown by a study of medical students during academic examinations: PBLs from blood samples collected at the time of the examination produced significantly less IFN and mediated significantly lower NK cell cytotoxicity than did PBLs from samples taken 6 weeks earlier (849). That the depression symptoms are more important than the stressful event per se in determining NK cell activity was shown in a study of 114 healthy undergraduate volunteers undergoing life change stress (850). The group of students reacting to the stress with psychiatric symptoms of depression (poor copers) had significantly lower NK cell activity than the group without symptoms (good copers) (850).

A clinically relevant cause of NK cell depression is surgical stress. A significant reduction of NK cell activity persists for 1–2 weeks after surgical operation (851–853). Studies in animal models showed that the surgical procedure and not the anesthesia was the cause of the NK cell suppression (853). Suppressor cells for NK cell activity after surgical stress have been demonstrated in both humans and mice (851, 853). The depressed NK cell activity after surgery could facilitate tumor metastasis spread (853).

In experimental animals depressed NK cell activity was observed in old rats subjected to isolation stress (854) and in mice subjected to restraint stress (855) or to rotation-induced stress (856). Transportation

stress in mice was sufficient to induce a significant decrease in NK cell activity lasting 24 hours and correlating with an increased plasma corticosterone level (857).

To test the hypothesis that opioid peptides released upon stress mediate the effect of the stress on the immune system, Shavit *et al.* (858-860) investigated the effect on NK cells of two types of inescapable foot-shock stress: (1) applied intermittently, causing analgesia that appears to be mediated by opioid peptides and learned helplessness, considered to be a model for human psychological depression and (2) applied continuously, inducing equally potent analgesia not involving opioids. The opioid but not the nonopioid form of stress suppresses the cytotoxic activity of NK cells in rats. The decrease in NK cell cytotoxicity by opioid stress is blocked by the opioid receptor antagonist naltrexone and is mimicked by systemic administration of morphine (858, 860). Morphine injected into the lateral ventricle of the brain suppresses NK cell activity to the same degree as a systemic dose three orders of magnitude higher, and this effect is also blocked by naltrexone (861). NK cell activity was unaffected by a morphine analog that does not cross the blood-brain barrier (861). These data implicate brain opiate receptors in the morphine-induced suppression of NK cell cytotoxicity. Morphine induces tolerance, i.e., repeated injections of morphine no longer result in suppression of NK cell activity, whereas foot-shock stress does not induce tolerance and is not prevented by the morphine-induced tolerance (859). The lack of tolerance and cross-tolerance with morphine might mean that the two effects on NK cells are mediated by different mechanisms or use different opiate receptors (859).

Corticotropin-releasing factor (CRF) administered as a single dose intraventricularly produced a dose-dependent suppression of rat splenic NK cell activity (862); however, neither systemic CRF nor CRF *in vitro* significantly altered NK cell activity. The NK cell-suppressive effect of CRF was antagonized by intraventricular, but not systemic, preadministration of a CRF antagonist (862). These data suggest that CRF released in the brain following stressful stimuli may have a role in controlling the modulation of NK cell cytotoxicity. The observed effect of CRF might be mediated by increased sympathoadrenal activity and/or activation of the pituitary-adrenal axis. Intraventricular administration of CRF produces an activation of sympathetic outflow and an acute increase in plasma concentrations of norepinephrine and epinephrine. Release of norepinephrine from sympathetic nerve endings innervating the spleen might then inhibit NK cell cytotoxicity (863). CRF might also act in part by stimulating the release of adrenocorticotropic hormone (ACTH) and β-endorphin from the anterior pituitary. However, the systemic

administration of CRF, which does not affect NK cell cytotoxicity, is able to induce ACTH and β-endorphin release from the pituitary gland. Furthermore, the suppression of NK cells is not blocked by the peripheral administration of CRF analogs, which prevent the effect on the pituitary mediated by the centrally administered CRF. ACTH and β-endorphin therefore do not appear to play a major role in the effect of CRF on NK cells *in vivo* (862).

Tail electrode shock, as well as foot shock, induces a transient depression of NK cell activity that is prevented by the opioid antagonists naloxone or naltrexone (864). However, β-endorphin injected *in vivo* increased NK cell activity, raising some doubt about the role of endogenous opioids in the suppression of NK cell activity in this system and about the specificity of naloxone and naltrexone as opioid antagonists (864). Indeed, in several *in vitro* studies, β-endorphin, Leu-enkephalin, and Met-enkephalin actually enhanced NK cell cytotoxicity and production of IFN-γ (865–869). However, Williamson *et al.* (870) have demonstrated activation of NK cells by β-endorphin in the range 10^{-11}–$10^{-8}M$, but inhibition of NK cell activity by this opioid in the range 10^{-17}–$10^{-13}M$. Thus, it is possible that β-endorphin is present *in vivo* after stress at the low concentration that induces suppression of NK cell cytotoxicity and is responsible for the inhibition. *N*-acetyl-β-endorphin, which has no opioid activity, does not affect NK cell activity (870). However, nonopioid fragments of β-endorphin enhance NK cell cytotoxicity, and the effect is blocked by naloxone (871). These data raise the possibility that the enhancement of NK cell cytotoxicity by endorphin fragments is not mediated through opioid receptors. The increase in cytotoxic activity of NK cells treated with β-endorphin is due to the increased number of target-binding cells and of cytotoxic cells among binders and to increased recycling capacity (872).

The evidence to date clearly shows that the activity of NK cells, as well as other cells of the immune system, is under the control of the central nervous system and is sensitive to various neuropeptides. There is little information as to whether NK cells, like other immune cells, can produce neuropeptides. The interaction between the nervous system and the NK cells is likely to have physiological and clinical relevance, although very little is known about the mechanisms of such interactions.

VII. NK Cells and Reproduction

The hormonal control of NK cell activity is suggested by alteration in NK cell cytotoxicity during the menstrual cycle and pregnancy. One study (873) reported a significant fall in human NK cell activity during

the periovulatory period, although another study (217) found no significant difference. In the mouse highest NK cell activity, corresponding to the time of the lowest metastatic potential of surgically removed mammary adenocarcinoma, occurs during the proestrus and estrus stages (874). During pregnancy, an NK cell depression is present from the first trimester to the postpartum period (875, 876). The mechanism of this depression is not clear, and both normal and decreased levels in the numbers of NK cells, in the NK cell cytotoxic potential, and in the recycling ability have been reported (877–880). The depression of NK cell cytotoxic activity in pregnancy correlates inversely with the level of 17β-estradiol in the sera of pregnant women. Treatment of mice with 17β-estradiol or diethylstilbestrol decreases NK cell cytotoxicity by decreasing the number of NK cells at the bone marrow level (881–883). Eventually, these hormones induce a condition of osteopetrosis, with destruction of the bone marrow environment and complete suppression of NK cell maturation (415). In some experimental conditions, however, 17β-estradiol treatment induced an activation of NK cells for the first 30 days, followed by NK cell depression (884). This early NK cell-stimulating effect of 17β-estradiol correlates with an estrogen-induced resistance to metastasis formation by B16 melanoma, an effect thought to be mediated by NK cells (884). *In vitro*, 17β-estradiol and diethylstilbestrol treatment of PBLs has been reported by some authors (885–887) to inhibit NK cell-mediated cytotoxicity, but not by others (888, 889).

Human, murine, and porcine embryos have been shown to recruit NK cells to the uterus (890–892). A modest increase of NK cell activity in the uterus was also observed in 17β-estradiol-induced pseudopregnancy, showing that hormonal regulation may play a role but not completely account for the sustained increase of NK cell activity in the decidua, which requires the presence of an embryo (891). In the human early pregnancy decidua 75% of the cells obtained by enzymatic digestion are of bone marrow origin (893). Immunohistological analysis showed that macrophages and $CD3^+$, $HLA\text{-}DR^+$ activated T cells predominate in the region, with prominent infiltration of the decidua by the trophoblast (894). In the area of the endometrium in which trophoblast invasion is not prominent or where it is still associated with endometrial glands or spiral arteries, the predominant cell type is that previously defined as endometrial granulocytes (895) and consisting of $CD2^+$, $CD3^-$, $CD5^-$, $CD38^+$, NKH-1/Leu-19^+, partly $HLA\text{-}DR^+$ cells (890, 894). These leukocytes tend to aggregate adjacent to degenerated endometrial glands or to spiral arteries (894). These granular lymphocytes are absent from term decidua. Flow-cytometric analysis of enzymatically

dissociated decidua cells showed that less than 10% of the cells were $CD3^+$, on average 40% were NKH-1/Leu-19^+, 30% were $CD2^+$, 10% were $CD16^+$, and over 50% were $CD38^+$ (893). Two-color analysis showed that the two major cell populations were NKH-1^+, $CD2^+$ and NKH-1^+, $CD2^-$, followed by $CD16^+$ cells (mostly expressing NKH-1/Leu-19 antigen at low density) and $CD3^+$ T cells (893). Most of these cells have an LGL morphology. Thus, it appears that more than 50% of the decidua cells in the first trimester of pregnancy have a phenotype compatible with that of NK cells. Similar results have been reported in the mouse system, although the phenotype of the cell was not extensively characterized (892, 896).

NK cells are unlikely to cause damage to the embryos because blastocysts or freshly dissociated 9.5-, 11.5-, and 14-day murine embryonic cells resist NK cell lysis as well as ADCC (892, 897). Human placental trophoblast cells are almost completely resistant to NK cell-mediated cytotoxicity, but are sensitive to ADCC (873). Lytic NK cells are absent from the decidua of beige mice and are reduced in the decidua of mice treated with anti-asialo-GM_1 serum (892), although pregnancy progresses normally in these animals, suggesting that NK cells do not play an essential role for a successful pregnancy. However, those data do not exclude the presence of NK cells defective in cytotoxic potential in the decidua which could mediate noncytotoxic functions of NK cells. It is possible that NK cells affect placentation by modulating the maternal immune response in the decidua or by producing lymphokines, such as IFN-γ, which have been shown to stimulate placental growth (898). Alternatively, decidual NK cells, through cytotoxic effects or by releasing cytolytic factors such as TNF, could participate in the necrosis of endometrial tissue, facilitating the trophoblast invasion. The striking predominance of NK cells in the decidual cellular infiltrate and the activated characteristics of these cells suggested by the expression of HLA-DR and CD38 antigens cannot be easily discounted as findings with little relevance for successful implantation. The absence of CD16 antigen from most of the cells with NK cell phenotype in the decidua might also mean that these NK cells are highly activated. CD16 antigen is known to be down-modulated following interaction of NK cells with immune complexes (132) or under conditions in which protein kinase C is activated (133). The high level expression of NKH-1/Leu-19 antigen on these cells is also an indicator of activation (100). Alternatively, the NKH-1/Leu-19^+, $CD16^-$ NK cells may represent relatively immature NK cells that are generated by rapid proliferation of NK progenitor cells in the decidua or that have recently migrated from the bone marrow. Although there is no information on whether the NK cells in the decidua

are cycling, the data suggest that the presence of an embryo induces an activation and localization of NK cells analogous to the localization observed at the site of virus infection (899). However, unlike during virus infection, systemic NK cell cytotoxic activity in early pregnancy is depressed and not stimulated.

The recruitment of NK cells may be mediated by products of the activated T cells observed in the decidua (894) or by soluble products from the endometrial cells or from the trophoblast. An alternative possible role of NK cells in the decidua is to suppress the immune response of the mother against the embryo. Decidua cells, especially at times of gestation later than those corresponding to the peak of NK cell activity, strongly suppress NK cell cytotoxic activity, as well as that of CTLs and ADCC effector cells (900, 901). Suppressor cells in the decidua also inhibit CTL generation. The suppressor cells have been identified as $Fc\gamma R^+$ non-T cell type, that can, however, be distinguished from classical NK cells because of lack of reactivity with anti-asialo-GM_1 serum, slower sedimentation rate, and presence in the decidua of beige mice (902). However, it is possible that the suppressor cells are NK cells at a stage of maturation or activation in which cytotoxic activity is low, and some of their antigenic and physical characteristics are different from those of resting mature NK cells with cytotoxic activity.

Although the studies described above have suggested a relative resistance of trophoblastic cells to the lytic effect of NK cells, other studies have shown that embryonal carcinoma cells are sensitive to NK cell-mediated cytotoxicity *in vitro* (530). It is therefore conceivable that if NK cell cytotoxic activity in the decidua is abnormally high, the trophoblast could be damaged. Indeed, some studies have suggested a direct correlation between NK cell activity and abortion rate. The NK cell activity of 50 women with threatened preterm delivery was found to be significantly higher than in 50 healthy pregnant women (903). In a murine model (CBA females × DBA/2J males) with a high spontaneous abortion rate, a significant correlation was found between NK cell infiltrates at 6–9 days and embryo abortion (904). In the same mouse model, poly I:C treatment was found to increase and anti-asialo-GM_1 serum treatment to decrease, the abortion rate in parallel with NK cell activity (905).

VIII. NK Cells and Hematopoiesis

Lymphocytes, mostly T cells, represent a small but significant proportion of bone marrow cells from healthy donors. Although NK cells originate and differentiate in the bone marrow (442), active mature

NK cells are almost entirely absent from the bone marrow of healthy donors (95). Alterations of T and NK cells in the bone marrow can be quantitative (increased number or change in the proportion of different subsets) or qualitative (activation of the cells). Although T and NK cells can produce both stimulating and inhibiting factors, bone marrow failure in one or more lineages is the hematopoietic condition most often associated with lymphocyte activation (906). The presence of inhibitory lymphocytes may represent a primary autoimmune mechanism, or they may be generated as a reaction to a pathogenic stimulus, e.g., infection or malignancy, with a secondary effect on hematopoietic cells. In some patients, the clonal or malignant expansion of a lymphocyte population with inhibitory activity is responsible for the failure of other hematopoietic cells. Lymphocytes may act directly on progenitor or stem cells or affect other accessory cell types required for growth factor production. Inhibition by lymphocytes may require direct cellular contact or be mediated via soluble factors.

A. Experimental and Clinical *in Vivo* Evidence for a Role of NK Cells in Regulation of Hematopoiesis

A role for NK cells in hematopoietic homeostasis was originally suggested by the pioneering studies by Cudkowicz and collaborators (361, 907, 908) on hybrid resistance to parental bone marrow transplantation in irradiated mice. Parental hematopoietic or lymphoid grafts do not survive in lethally irradiated F_1 hybrids, even though these animals are universal recipients of grafts of other types of parental tissue (907). The genetic control of hybrid resistance contrasts with the classical transplantation studies which show that graft compatibility rests predominantly on multiple genetic determinants of cellular antigens inherited codominantly: the histocompatibility (H) antigens. The F_1 hybrid anti-parent reaction has been explained by assuming the existence of a class of noncodominant genes, designated Hh for hematopoietic (or hybrid) histocompatibility, with tissue distribution restricted to hematopoietic cells (907). By transplanting across allogeneic and xenogeneic barriers, using recipients in which the T cell response has been abrogated by irradiation, it was possible to demonstrate an Hh-controlled allogeneic and xenogeneic resistance to hematopoietic cells that shares most of the properties of hybrid resistance (909). The characteristics of the effector cells mediating hybrid resistance (e.g., radioresistance, age of maturation, bone marrow dependence, thymus independence, sensitivity to split-dose irradiation, and lack of immunological memory) suggested their identity with NK cells (361, 908). In the mouse both hybrid resistance and NK cell activity are under similar genetic

control (374) and are abrogated *in vivo* by treatment with antisera recognizing NK cells (910, 911); hybrid resistance is reduced in NK-deficient beige mice (912), and the ability to reject bone marrow in a genetically restricted way is adoptively transferred by clones with NK cell activity (913). However, the list of properties shared between the cells responsible for hybrid resistance and NK cells does not include the single most pertinent property of hematopoietic resistance, i.e., its immunogenetic specificity. The genetic restriction of natural hybrid resistance has been reproduced in an *in vitro* system in which purified murine F_1 NK cells inhibit parental granulocyte-macrophage colony-forming units (CFU-GMs) (914), although a lower but significant suppression was also observed against syngeneic progenitor cells (914, 915). The genetic specificity has been shown by *in vivo* experiments of competitive inhibition to reside at the effector cell level (916). A possible role of regulatory radioresistant T cells or of natural antibodies in determining genetic specificity of hybrid resistance has been proposed, but these models do not account for all properties of hybrid resistance (917).

In vivo NK cells suppress hematopoietic progenitors in mice experimentally infected with lymphocyte choriomeningitis virus (LCMV) (918). Adult mice injected intraperitoneally with LCMV undergo a relatively mild disease followed by marked immunological and hematological dysfunction (919, 920). During the first week of infection, there is a profound suppression of spleen CFUs (CFU-Ss) and CFU-GMs (919, 920). Erythropoiesis, as measured by ^{59}Fe uptake into hematopoietic tissue, is also markedly suppressed. After day 10 of infection, CFU-S and erythropoiesis return to levels higher than normal in spleen, whereas hematopoiesis remains depressed for over 3 weeks in bone marrow. The *in vivo* infection of mice with LCMV results in IFN production and increased NK cell activity in spleen and bone marrow (921), accompanied by the appearance of NK blasts and proliferation of NK cells (922). In the infected mice, NK cell activity and tissue distribution in the animals correlate with hematopoietic dysfunction (918) although the long-lasting bone marrow defect cannot be completely explained by the effect of NK cells. NK cell activity is detected in the bone marrow during LCMV infection, suggesting that the depression of hematopoiesis at early times during infection might be attributed to NK cells (918). The NK cells in bone marrow have the antigenic phenotype of immature NK cells, suggesting that either increased local production or delayed migration of NK cells from bone marrow accounts for the increased cytotoxic activity (918). An adoptive transfer system was used to show that irradiated LCMV-infected mice reject syngeneic bone marrow and that this resistance is almost completely abolished by treatment with anti-asialo-GM_1

antiserum, which abolishes NK cell activity (918). These experimental observations in LCMV-infected mice demonstrated that *in vivo* activated NK cells can suppress growth and proliferation of syngeneic hematopoietic progenitor cells and that this suppression can occur in organs, such as the bone marrow, in which NK cell-mediated cytotoxicity is normally low.

The possibility that NK cells play an important regulatory role in physiological hematopoiesis, at least in extramedullary sites, is strongly suggested by data showing that CFU-GM precursors in the spleen but not in the bone marrow are increased severalfold in normal mice depleted of endogenous NK cells by chronic treatment with antibody NK-1.1 (923).

In humans several clinical situations of bone marrow depression are associated with the presence of activated lymphocytes (906). In many cases the activated lymphocytes capable of hematopoietic suppression are T cells, mostly of the suppressor/cytotoxic $CD8^+$ subset (924), that express HLA-DR and CD25 activation antigens (925). The identification of NK cells as responsible for bone marrow suppression in human pathology has been difficult because of the ambiguity of distinctive characteristics between NK cells and activated T cells. The LGL morphology, typical of resting NK cells, is often presented by activated T cells, especially $CD8^+$ T cells. In several early studies antibody HNK-1/Leu-7 was used as a reagent for NK cells (146). The Leu-7 antigen is present in normal PBLs on a proportion of NK cells and in a small subset of T cells (95), and in patients with activated T cells a large proportion of Leu-7^+ T cells is often observed. The low-affinity FcγR recognized by anti-CD16 antibodies (95) is also expressed on T cells from some patients. Cells bearing the receptor for SRBCs (CD2 antigen) and FcγR, often referred to as Tγ cells, in normal peripheral blood correspond to the NK cell subset (95). However, in several patients with bone marrow failure (e.g., EBV infection, pure RBC aplasia during chronic lymphocytic leukemia, and LGL lymphocytosis), $CD2^+$ cells expressing FcγR and/or CD16 antigens have characteristics of T cells, i.e., they express the TCR and the TCR-associated CD3 antigen. Approximately 10% of the patients with LGL lymphocytosis present cells with $CD3^-$, $CD16^+$, $CD2^+$, $CD8^+$ or $CD8^-$, high spontaneous cytotoxic activity, and no rearrangements in the TCR genes (423, 430–432). These cells have phenotypic and functional characteristics identical to those of peripheral blood NK cells. Chan *et al.* (437) observed that nine patients with $CD3^+$ LGL lymphocytosis presented neutropenia, whereas two patients with $CD3^-$ LGL presented no abnormalities in granulopoiesis. However, other recent studies described patients with $CD3^-$, $CD16^+$ LGL lymphocytosis associated with neutropenia and anemia (438, 926, 927). Cells from two

of these patients were studied *in vitro* and were shown to inhibit proliferation/differentiation of progenitor cells (926, 927).

Expansion of Leu-7$^+$ LGLs was also reported in patients with Felty's syndrome (neutropenia, arthritis, splenomegaly) and with adult-onset cyclic neutropenia (928, 929). In the patients with Felty's syndrome the Leu-7$^+$ LGLs are CD3$^+$ and of T cell origin, but, unlike in LGL lymphocytosis, the CD3$^+$ cells express the CD5 antigen and show polyclonality of TCR gene rearrangement (928, 930). On the other hand, two of three patients with cyclic neutropenia described by Loughran *et al.* (929) showed expansion of LGLs with the typical phenotype of NK cells.

B. Inhibition of *in Vitro* Hematopoiesis by Human NK Cells

It has been hypothesized that the *in vivo* role of NK cells might be surveillance of primitive cells (529). The proportion of NK cells is high in blood, spleen, and liver, but low in bone marrow and thymus. Normal primitive cell types with significant susceptibility to NK cell lysis *in vitro* can be found in bone marrow and thymus (36, 931–933).

Because progenitor cells represent only a very small proportion of bone marrow cells and their purification has presented serious technical difficulties, it has been very difficult to directly analyze a cytotoxic effect of NK cells on progenitor cells. Most of the *in vitro* evidence for a role of NK cells in inhibiting hematopoiesis comes from experiments testing the ability of purified NK cell preparations to suppress proliferation and differentiation of CFUs. Several early studies suggested that human lymphocytes with some characteristics of NK cells (e.g., light density and expression of FcγR and E receptor) inhibit both autologous and allogeneic bone marrow CFUs, that the inhibition was enhanced by pretreatment of NK cells with IFN, and that NK cell-sensitive target cells competed for the inhibition (934–937). The inhibitor cells were resistant to 10 Gy irradiation but required several hours of contact with the bone marrow cells before plating in semisolid medium, in order to mediate maximum inhibition (936). Bone marrow-derived CFU-GMs and erythrocyte CFUs (CFU-Es) are maximally inhibited by NK cells (668, 934–939), whereas inhibition of erythrocyte burst-forming units (BFU-Es) was observed in only one study (939) using HNK-1/Leu-7$^+$ cells. Two studies have shown that peripheral blood-derived CFU-GMs are not inhibited, but rather are stimulated by NK cells (122, 940).

A possible role for NK cells in inhibiting not only normal hematopoietic progenitor cells but also clonogenic growth of leukemia cells was suggested by Beran *et al.* (941), who found that allogeneic Percoll-purified NK cells prevented colony formation by the blasts of three patients with acute myeloid leukemia. The anti-leukemia cell effect

of NK cells was boosted by pretreatment of effector cells with IFN. Interestingly, as observed for NK cell-mediated killing of target cell lines (34, 503), IFN treatment of the leukemic cells rendered them resistant to the suppressive effect of NK cells (941).

Degliantoni *et al.* (668, 938) showed that the peripheral blood cells that spontaneously suppress bone marrow hematopoietic colonies have the exact phenotype of NK cells (i.e., $CD16^+$, $NKH\text{-}1^+$, $CD3^-$, $CD5^-$, $CD4^-$, HLA-DR^-, mostly $CD2^+$, and, in part, $CD8^+$ and $HNK\text{-}1^+$). The suppressive effect of these cells was increased by pretreatment with IFN-α. Herrmann *et al.* (942) have recently analyzed the ability of human NK cell clones and $CD3^+$ T cell clones with NK cell-like cytotoxic activity to suppress *in vitro* hematopoiesis. The NK cell clones did not promote hematopoietic colony growth, and individual NK cell clones suppressed subpopulations of progenitor cells in a heterogeneous but clonally stable manner. The generation of the inhibitory effect required cell-to-cell contact, and maximum inhibition was observed after 8–18 hours of preincubation.

The possibility that NK cells residing in the bone marrow have an inhibitory effect on colony formation was suggested by studies showing a significant increase in the number of CFUs when NK cells were removed from bone marrow preparations using anti-CD16 antibodies and complement (943). However, in most studies the bone marrow used as the source of CFUs is obtained by aspiration and is likely to be contaminated by peripheral blood, making it difficult to establish the origin of the NK cells.

The *in vivo* relevance of the observed reactivity of NK cells *in vitro* against syngeneic progenitor cells is suggested by studies of cells from a patient with aplastic anemia that twice failed to reconstitute after engraftment with bone marrow of an identical twin (944). The patient's peripheral blood cells, with characteristics of NK cells (e.g., LGLs, $CD4^-$, $CD8^-$, cytotoxic for K562 cells) caused marked inhibition of syngeneic CFU-GM colonies (944), suggesting that NK cells might be involved in both the pathogenesis of the anemia and the rejection of the graft.

C. Role of Soluble Factors in the Modulation of Hematopoiesis by Lymphocytes

NK cells produce various types of factors, including growth factors, which affect hematopoiesis. Stimulated highly purified NK cells have been shown to produce high levels of GM-CSF and, in certain conditions, M-CSF and IL-3 (799). GM-CSF and/or IL-3 could account for the burst-promoting activity produced by NK cells (945). NK cells have

also been shown to support megakaryocyte colony formation by producing a soluble CSF (945, 946); because IL-3 has the same activity, it is not clear whether IL-3 accounts for the activity produced by NK cells, or instead, whether NK cells produce a separate factor.

Inhibitory factors released by activated T cells and NK cells have also been shown to be responsible for hematopoietic suppression *in vitro* and possibly *in vivo* (947, 948). The effect of NK cell supernatant fluids on *in vitro* colony formation is a balance between these inhibitory and stimulatory activities. However, most assays of colony formation in the presence of optimal concentrations of exogeneously added CSF preferentially detect inhibitory activities, whereas, in the absence of added CSF, stimulatory activity can be observed (949).

Degliantoni *et al.* (668, 938) showed that purified NK cells produce colony-inhibiting activity (NK-CIA) when cocultured for several hours with NK-sensitive cells (such as K562 cells) or with allogeneic or autologous bone marrow cells, but not with NK-insensitive cells (such as Raji cells). HLA-DR^+ bone marrow cells, highly enriched for hematopoietic progenitor cells, induce NK-CIA production, whereas HLA-DR^- cells, depleted of precursor cells, fail to do so, suggesting that NK cells produce NK-CIA following direct interaction with the progenitor cells. The specificity of inhibition of hematopoietic colonies by NK-CIA and by NK cells was almost identical: Both inhibited CFU-GEMMs, CFU-Es, and CFU-GMs on day 14, but not BFU-Es or CFU-GMs day 7 (938). NK-CIA was synergistic with IFN-γ in inhibiting CFU-GMs on day 14; NK-CIA and IFN-γ together but not separately, also inhibited CFU-GMs on day 7 (668). The NK-CIA concentration in the supernatant fluid was sufficient to account for the inhibition of colony formation observed when NK cells were added directly to the bone marrow cells used for colony formation, although the contribution of a direct cytotoxic effect of NK cells on progenitor cells to the observed inhibitory effect cannot be ruled out. NK-CIA-containing supernatants did not contain significant amounts of IFN-α or -γ, and the NK-CIA activity was not inhibited by antibodies to IFN (668). NK-CIA inhibition of colony formation was efficiently abolished by monoclonal antibodies to TNF but not lymphotoxin (LT) (668). The NK-CIA-containing supernatants have low (0.1–10 U/ml) TNF activity, as evaluated by biological assay (cytotoxicity on actinomycin D-treated mouse L cells) or by radioimmunoassay (260). Such levels of TNF were sufficient to account for the observed inhibition of colony formation, as determined by using recombinant TNF (668, 950, 951).

Purified recombinant TNF as well as LT inhibit CFU-GEMMs, BFU-Es, and CFU-Es with similar efficiency ($\sim$50% inhibition with

1 U/ml), and the inhibition is augmented by IFN-γ (951). The fact that homogeneous TNF but not supernatants from NK cells containing TNF inhibited BFU-E colonies is probably due to the fact that NK cells produce burst-promoting activity, masking the inhibition by TNF (945). Both TNF and LT poorly inhibit CFU-GMs but strongly synergize with IFN-γ in inhibiting this colony type (951). When NK and T cells simultaneously produce IFN-γ and TNF or LT, the ability of the supernatants to inhibit CFUs, because of this synergistic effect, might be almost completely abolished by anti-IFN-γ, leading to the mistaken conclusion that IFN-γ alone is responsible for the inhibition. In the study by Herrmann *et al.* (942) NK cell clones that produced both IFN-γ and NK-CIA activity inhibited erythroid and myeloid colonies, including CFU-GMs on day 7. Anti-IFN-γ monoclonal antibodies prevented the inhibition of CFU-GMs on day 7, but not of other colony types, suggesting that the inhibition was mediated by a factor (possibly TNF) acting synergistically with IFN-γ. Cells from several LGL lymphocytosis patients have been shown to produce IFN-γ (432, 438). In one case of $CD3^-$, $CD16^+$ LGL lymphocytosis, IL-2-stimulated LGL produced both IFN-γ and a CIA that was only partially abolished by anti-IFN-γ antibodies, also suggesting a synergistic effect between IFN-γ and other factors, possibly cytotoxins (438).

Because progenitor cells in peripheral blood and in bone marrow are not qualitatively different, it is difficult to interpret the data showing that bone marrow CFUs but not peripheral blood CFUs are inhibited (122, 940). However, it was recently shown that removal of NK cells from the peripheral blood of patients with β-thalassemia results in increased CFUs but that the effect of NK cell removal is abolished if adherent cells are removed (952). These data suggest that the inhibition of CFUs mediated by NK cells in the peripheral blood of the patients requires interaction with adherent cells. It is possible that the $HLA\text{-}DR^+$ cell population in bone marrow shown to induce NK-CIA/TNF formation by NK cells (938) contains a stromal or hematopoietic cell population in addition to precursor cells. This stromal/hematopoietic cell type, but not the precursor cells, could interact with NK cells and induce NK-CIA/TNF production. The absence of this accessory cell type from nonadherent preparations of peripheral blood mononuclear cells might explain the inability of NK cells to suppress CFUs from peripheral blood.

Both experimental and clinical observations support the possibility that NK cells are the cellular mediators of certain types of pathological dysregulation of hematopoiesis. *In vitro* models have offered insights into the mechanisms of interaction of NK cells with hematopoietic progenitor cells. NK cells probably directly interact with progenitor cells, with a

mechanism of specificity still unknown, and are triggered to produce various soluble mediators. NK cells can produce factors with both enhancing and inhibiting activity on hematopoiesis. The activity of NK cells and their ability to produce cytotoxins are also regulated by other cell types through factors such as IL-2 and IFN-α.

The evidence for a role of NK cells in maintaining physiological hematopoietic homeostasis is much less compelling. However, if, as in many other systems, the pathological aspects of NK cell functions are interpreted to be an exaggeration of the physiological functions of this cell type, a role of NK cells in hematopoietic homeostasis can be assumed. The observation that depletion of NK cells *in vivo* does not affect bone marrow hematopoiesis, but determines a significant increase in the number of progenitor cells in the spleen (923), suggests the possibility that NK cells are mostly involved in the regulation of extramedullary hematopoiesis. This possibility is also compatible with observations in the hybrid resistance system (912), with NK cell organ distribution, and with the localization of their effect against metastatic diffusion of tumors or parasite infection (23).

D. NK Cells and Graft-versus-Host Reaction

The pathogenesis of acute graft-versus-host disease (GVHD), a major complication of allogeneic bone marrow transplantation, remains obscure. The identity of the effector cells involved in acute GVHD is still controversial, and CTLs, NK cells, and a decreased activity of suppressor T cells have all been implicated, but none of these cell types has been definitively incriminated in the production of target cell injury *in vivo*.

In humans Lopez *et al.* (953-955) found an association between high pretransplantation NK cell activity in the recipient against HSV-1-infected target cells and incidence of GVHD after bone marrow transplantation. However, in other studies, no correlation was found with NK cell cytotoxic activity against K562 target cells (953, 956, 957) or, in one study (957), against HSV-1-infected target cells. The failure to reproduce the original finding of an association between NK cell activity in the recipient and GVHD is probably due to the number of different factors that affect measurement of NK cell activity *in vitro*, making it impossible to use this activity as a clinical prognostic indicator of GVHD. However, the data of Lopez *et al.* (953-955) are in agreement with observations in experimental animals, as detailed below, and represent suggestive important evidence of a role for NK cells in both the inductive and effector phases of GVHD.

Dokhelar *et al.* (467) reported further evidence supporting a role for NK cells in human GVHD by showing that the occurrence of acute

GVHD was associated with an early appearance of maximal NK cell activity within 2–4 weeks of transplantation, whereas in patients without acute GVHD NK cell activity was restored later. More direct evidence of NK cell participation is represented by *in situ* analysis during GVHD of human rectal mucosa (958) and skin (959), in which cells with immunohistochemical features of NK cells were detected. In the murine model of GVHD induced by bone marrow transplantation between strains differing only in the minor histocompatibility antigens, Guillen *et al.* (960) demonstrated that the preponderant mononuclear cells in GVHD lesional skin have phenotypic characteristics of NK cells. These cells have the morphology and ultrastructure of LGLs with typical vesicles and PTAs, express asialo-GM_1, Thy-1, and CD11b (MAC-1) antigens, but are mostly Ly-1 and Ly-2 negative. Membrane association was observed between LGLs and degenerating keratinocytes, including apposition of cell membranes and invagination of elongated microvilli of mononuclear cells into adjacent degenerating keratinocytes (960). On the basis of this association and a granule morphology suggesting discharge or dissolution of the granule contents, it has been postulated that the NK cells are directly cytotoxic for the keratinocytes (960). However, it has recently been reported in a similar mouse model that anti-TNF antibodies *in vivo* completely prevent acute GVHD (961). Because activated NK cells are powerful producers of TNF (78), it is possible that the tissue necrosis observed is mediated by TNF and not by NK cell-mediated cytotoxicity. Alternatively, TNF may act as an immune potentiating cytokine that enhances the cytotoxic/necrotic effect of NK cells and, possibly, of macrophages and neutrophils.

Although the data presented above provide compelling evidence for a major role of NK cells in the effector phase of GVHD, the role of NK cells in the inductive phase and their host or donor origin remain controversial. In experimental animals (962) and possibly in humans (963) elimination of mature T cells from the bone marrow graft prevents GVHD. A role for transplantation antigen-specific T cells in the initiation of most cases of GVHD is certain. NK cells might be recruited by the products of T cells as effector cells, but they might also participate in the induction phase, providing necessary help for the T cell response (see Section X). Various experimental systems have been utilized to demonstrate the requirement of host or donor NK cells in GVHD. When $+/bg$ (normal NK cell activity) or bg/bg (deficient NK cell activity) mice were used as the host or donor of bone marrow cells, early splenomegaly and moderate B cell suppression were observed in all of the combinations. However, $+/bg$ but not bg/bg bone marrow cells were able to induce severe GVHD with histopathological lesions and profound B and T cell suppression in either bg/bg or $+/bg$ recipients (964). These

results suggest that donor NK cells rather than host NK cells play an active role in GVHD-associated tissue damage and long-term immune suppression. Elimination of either asialo-GM_1^+ (965) or NK-1.1$^+$ (966) cells from transplanted bone marrow did not prevent GVHD, indicating that mature donor NK cells were not required. However, when donor mice were stimulated with *in vivo* allogeneic immunization, maturation of a proportion of lymphocytes, possibly NK progenitors, from asialo-GM_1^- to asialo-GM_1^+ was observed (965). When bone marrow from these immunized animals was used for transplantation, treatment of the animals with anti-asialo-GM_1 serum before marrow harvest prevented GVHD in the recipients (965). Thus, both mature and precursor NK cells in the bone marrow graft might generate GVHD.

Other studies have shown that treatment of the recipient with anti-asialo-GM_1 serum prevents GVHD following semiallogeneic bone marrow transplantation in irradiated (967) or unirradiated (968) animals. In animals treated with anti-asialo-GM_1 serum the anti-host CTL response (967) and the enhancement of NK cell activity (968) found in the control grafted animals were suppressed.

The most likely interpretation of these apparently contradictory results is that radioresistant NK cells are required in the host to provide necessary helper function for the generation of alloantigen-specific CTLs. This helper function of NK cells might be present in the noncytotoxic NK cells from beige mice, as suggested by the development of GVHD in *bg/bg* mice transplanted with *+/bg* bone marrow (964). Probably secondary to CTL activation, donor NK cells or NK progenitor cells are activated and induced to proliferate, generating the early and elevated reconstitution of NK cell activity after transplantation. These activated NK cells probably represent the effector cells of GVHD without antigenic specificity. The lack of antigenic specificity of this phase of the GVHD is elegantly demonstrated by experiments in which a graft of fetal intestine syngeneic with the bone marrow donor was implantad under the kidney capsule of mice undergoing GVHD (969). Although the intestine should not have been recognized by the anti-host CTLs, it was nonetheless rapidly infiltrated by lymphocytes and presented the same pathological aspects (e.g., villus atrophy and crypt hyperplasia) observed in the recipient intestine.

IX. Antimicrobial Activity of NK Cells

A. Antiviral Activity of NK Cells

A central role for NK cells in the defense against virus infection in humans is strongly suggested by the prevalently viral pathology in the

few patients who have a selective absolute deficiency of NK cells (375, 377). These patients show frequent infections with varicella zoster, CMV, EBV, and other viruses. Unlike patients with X-LPD, in whom the NK cell defect is subsequent to EBV infection and might be induced by the virus (339), the NK cell-deficient patients had a history of repeated viral infection before EBV infection (375).

NK cells, together with IFN and other natural resistance mechanisms, represent the first line of defense of the organism against infection by certain viruses, before humoral and cellular effectors of adaptive immunity are activated. During virus infection, an NK cell response, which usually peaks at 3 days postinfection, is followed by a CTL response, which peaks at 7-9 days postinfection (921). Mice acutely infected with LCMV are characterized by high levels of virus-induced IFN and NK cell activity in spleen, peritoneum, liver, lung, bone marrow, and peripheral blood (17, 899, 918, 921, 970, 971). The increase in NK cell activity is due to an absolute increase in the number of NK cells, originating *de novo* from the bone marrow, as indicated by the prevention of activation of NK cells by HU treatment (922, 972). NK cells in the infected mice have blast morphology, are of lighter density than are those in control mice, and are replicating, as shown by experiments combining single-cell cytotoxic assay and autoradiography using NK cells pulsed with[^{3}H]thymidine (972). Similar *in vivo* activation and proliferation of NK cells are observed when mice are treated with IFN or with IFN-inducers such as poly I:C (734), suggesting that the effect of virus infection on NK cell blastogenesis *in vivo* is mediated through IFN induction.

Although virus-infected mice show systemic activation of NK cells, there is also a preferential localization of NK cells in the infected organs, as shown by higher peritoneal accumulation of LGLs when viruses are injected intraperitoneally rather than intravenously and by higher liver accumulation of NK cells observed with infection by hepatotropic viruses than by nonhepatotropic viruses (899, 973). Production of chemotactic factors at sites of virus replication is at least partially responsible for NK cell or LGL accumulation at these sites, as suggested by the presence of *in vitro* chemotactic activity for NK cells and other cell types in the washout fluid from the peritoneal cavity of virus-infected animals (922).

LCMV infection is very efficient in inducing NK cell activation *in vivo*. However, experimental evidence suggests that NK cells do not play a primary role in protecting the mice against this virus (974-976). The inflammatory exudate found in the cerebrospinal fluid of mice after intracerebral infection with LCMV contains a substantial population of NK cells in addition to CTLs; however, various experimental protocols, including adoptive cell transfer, suggest that NK cells, even if they participate in the inflammatory process, are not uniquely required

for the induction of neurological symptoms (977). On the other hand, the severity of the encephalopathy induced in mice by intracerebral injection of influenza virus is significantly reduced by elimination of NK cells *in vivo* using anti-asialo-GM_1 serum (978). Thus it appears that under conditions in which NK cells are unable to prevent the virus infection, they might participate in the pathogenic process itself.

CMV infection of the mouse is presently the system with the most convincing evidence that NK cells play a role in resistance to virus infection *in vivo*. This was shown by correlative experiments in different mouse strains, by altering NK cell activity *in vivo* with stimulators and inhibitors, including treatment with anti-asialo-GM_1 antiserum, and by adoptive transfer experiments (976, 979, 980). Injection of anti-asialo-GM_1 antiserum up to the third day of infection increases the virus titer up to 1000-fold (980). However, systemic treatment with anti-asialo-GM_1 serum does not exacerbate infection by mouse CMV administered intranasally (980). This phenomenon, originally interpreted as evidence against a role for NK cells in protection against viral infection in the lung, is now known to be due to a compartmentalization of lung NK cells, which respond poorly to systemic stimuli but can be efficiently activated in their antiviral function by local stimulation (315, 981).

It is possible to speculate why NK cells are very efficient against certain viruses but not others. IFN production is a constant feature of virus infection and IFN renders tissue cells resistant to the lysis mediated by both resting and activated NK cells (34, 503, 570). Because cells infected by most viruses are not protected against NK cells by IFN, due to the inhibition of host RNA and protein synthesis, IFN protection of normal but not infected target cells was proposed as a major mechanism by which NK cell cytotoxicity is directed toward virus-infected cells and spare uninfected cells (34, 569). This theory predicted that NK cells would not be effective *in vivo* against viruses that do not shut off host RNA and protein synthesis during cell infection. Cells infected by such viruses would be protected by IFN and therefore not lysed by NK cells. The work of Welsh and collaborators (511, 570, 976, 982) has provided supportive evidence for this hypothesis by showing that (1) normal cells such as thymocytes are protected *in vivo* by IFN against NK cell cytotoxicity during LCMV infection and (2) infection of target cells with viruses sensitive to NK cells *in vivo*, such as mouse CMV, prevents the protective effect of IFN, whereas LCMV does not.

The role of NK cells in the defense against infection by HSV-1 in mice is controversial. Original data that anti-asialo-GM_1 serum suppresses resistance to HSV-1 infection (983) were challenged by the observation that the antiserum suppresses both NK cell activity and IFN production

(984). The use of lower concentrations of anti-asialo-GM_1 serum that were able to block NK cell activity but not IFN production failed to confirm a role of NK cells against HSV-1, and adoptive transfer experiments were also inconclusive in supporting a role for NK cells (984). More recently, however, adoptive transfer of purified NK-1.1$^+$, asialo-GM_1^+ NK cells in cyclophosphamide-treated mice has been shown to induce protection against HSV-1 infection, providing direct evidence for a role of NK cells in protection against development of fatal infection in mice (985). These data also point to the need for caution in interpreting studies that fail to demonstrate a role for NK cells against other viruses. Several adaptive and nonadaptive mechanisms of resistance to virus infection are simultaneously active in the organism. When one mechanism fails or is suppressed, it seems reasonable to expect that other mechanisms might compensate, making it difficult to dissect the role of a particular mechanism. In this respect, it is interesting that the IFN titer *in vivo* in mouse CMV-infected mice is higher when NK cells are ablated by anti-asialo-GM_1 serum treatment (976). Experimental evidence, even if not always conclusive, suggests a role for NK cells in the defense against infection by mouse hepatitis virus, vesicular stomatitis virus, influenza virus, togaviruses, retroviruses, poxviruses, and also non-enveloped viruses such as coxsackie B and encephalomyocarditis viruses (982).

Studies of NK cell activity during virus infection in patients are rare, and in most cases the available information is not based on sufficiently standardized assays. Enhanced NK and K cell activity has been observed during several acute virus infections (986–992). An increase in NK cell activity was observed in renal transplant patients during CMV infection (986), and a significant correlation was found between fatal CMV infection and failure to develop NK cell activity in immunosuppressed bone marrow transplant recipients (993). A correlation has also been observed between susceptibility to HSV-1 infection and low NK cell activity against HSV-1-infected target cells in newborns and in patients with acquired immunodeficiency syndrome (AIDS) (992, 994).

In vitro, human NK cells efficiently lyse virus-infected cells. Cytotoxicity against HSV-1-infected target cells was originally shown to be due either to an ADCC mechanism induced by minimal concentrations of antibodies produced *in vitro* (995) or to cross-linking mediated by immune complexes or aggregated Igs between FcγR$^+$ effector cells and the FcR induced by HSV-1 infection on the target cells (996). However, antibody-independent natural cytotoxicity was demonstrated on mumps-infected target cells (997), an observation subsequently extended to target cells infected by a variety of viruses (34, 998, 999), demonstrating that

(1) sensitivity of virus-infected target cells to NK cell cytotoxicity was not significantly different from that of noninfected target cells, but infected cells induced activation of NK cells, resulting in increased killing of the infected (and uninfected bystander) target cells starting after 3–4 hours of culture, (2) activation of NK cell cytotoxicity was concomitant with the production of IFN-α by the PBL preparations used as a source of NK cells, and (3) the IFN-α released into the supernatant was able to stimulate the cytotoxic activity of fresh PBLs. These data served to identify IFN-α as the major factor responsible for the enhanced NK cell cytotoxicity against virus-infected target cells.

An IFN-independent cytotoxic mechanism was described particularly for target cells infected with paramyxovirus and myxoviruses. Lysis against these target cells can be blocked by antibodies to viral glycoproteins, specifically hemagglutinin, and purified glycoproteins can activate the effector cells (999–1002). Antibodies to the glycoproteins do not block the cytotoxicity if added after the glycoproteins have activated the effector cells. This type of cytotoxicity has been defined as virus-dependent cellular cytotoxicity (VDCC) (1003) and has characteristics quite different from those of the NK cell-mediated lysis of virus-infected target cells. The increase in cytotoxicity is observed within 3–4 hours after the treatment of PBLs with glycoproteins, at a time in which NK cell-mediated killing of virus-infected target cells is usually not observed (1001). This type of killing is reminiscent of lectin-dependent cytotoxicity and both NK cells and $CD3^+$ T cells are able to mediate VDCC and lectin-dependent killing (1004–1008). In other experimental models target cells infected by herpesviruses have been shown to be lysed mostly or exclusively by NK cells (510, 1009, 1010). Interestingly, the nature of the infected target cells seems to indicate which type of effector cells is involved: mumps virus-infected Chang target cells were lysed only by $CD3^-$ effector cells, whereas mumps virus-infected T24 cells were lysed by both $CD3^-$ and $CD3^+$ effector cells (1007). This observation may indicate different mechanisms of lysis that may or may not involve IFN. In the murine system HSV-1-infected YAC-1 cells are lysed by NK cells activated by a mechanism involving exclusively IFN activation, whereas HSV-1-infected WEHI-164 cells are lysed by NC cells that are not stimulated by IFN (1011). The possible involvement of NC cells is interesting because the cytotoxicity of NC cells is mostly mediated by TNF, and it was recently shown that NK cell clones can also lyse vesicular stomatitis virus (VSV)-infected target cells through the release of TNF (1012).

It is difficult to evaluate the relative participation of VDCC and IFN-mediated activation of NK cells in the cytotoxicity of PBLs against virus-

infected target cells. Casali and Oldstone (1013) have shown that lysis of measles-infected target cells occurs in two phases. The first phase occurs within 4 hours and is blocked by antibody to the hemagglutinin glycoprotein but is not blocked by antibody to IFN; the second phase of lysis occurs betwen 8 and 16 hours and is inhibited by antibody to IFN. Because enhanced lysis of virus-infected target cells in most experimental systems is not detectable until 4 hours (Fig. 3), it is possible that VDCC requires a very high density of viral glycoproteins on the target cell surface and does not play a major role in most *in vitro* systems.

Activation of human NK cells has also been shown with influenza hemagglutinin in an IFN-independent system (1014). Those results were confirmed using recombinant influenza virus proteins (nonstructural protein 1, alone or fused with the hemagglutinin or matrix protein) but in this case, the activation of NK cells was shown to be mediated entirely by IFN-α induced by the virus proteins (1015).

The hypothesis that IFN is a major inducer of NK cell activation against virus-infected target cells has been challenged by several authors (512, 513, 1016-1019) on the basis that (1) antibodies against IFN do not block cytotoxicity, (2) IFN-activated NK cells are able to lyse virus-infected target cells more efficiently than uninfected ones, and (3) using as effector cells PBLs from normal donors or immunodeficient patients, there is no correlation between IFN titer in the supernatant fluid and cytotoxicity. Although the third observation can be easily explained by factors such as the variable response of PBLs of different donors to IFN stimulation, the characteristics of the dose-response curve for IFN-mediated NK cell activation, and the possibility of other mechanisms operating together with IFN, the underpinnings of the first two points are less obvious. The inability of anti-IFN antibodies to inhibit cytotoxicity might rest in the use of the antibodies at concentrations too low to efficiently inhibit, before NK cell activation, the high concentrations of IFN expected to be present in the intercellular space in the cell pellet. Also, both the antiviral and the NK cell-activating activities of IFN can be transferred directly from cell to cell, without secretion of IFN into medium containing anti-IFN antibodies (1020, 1021). The ability of IFN-stimulated NK cells to lyse virus-infected cells more efficiently than uninfected cells suggests that virus-infected target cells are intrinsically more sensitive to NK cells. These results have been reported by some laboratories (1016, 1018), whereas others found a similar or lower lysis of infected versus uninfected target cells when optimally stimulated NK cells were used (79, 511-513). The interpretation of these results is complicated by the facts that maximal activation of NK cells may not always be obtained, that IFN produced during an 18-hour cytotoxic assay further

increases or at least maintains the cytotoxic activity of the IFN-treated PBLs, and that IFN, when added to the assay, protects the uninfected but not the infected target cells from lysis, mimicking the higher sensitivity of the virus-infected target cells.

Because the cells that produce IFN-α in cultures of PBLs and virus-infected target cells share some characteristics with NK cells, e.g., similar density on a Percoll gradient, it was proposed that NK cells produce IFN-α and therefore stimulate themselves with an autocrine mechanism (821). However, it was found that the major type of IFN-α-producing cells in response to CMV, HSV-1, and influenza virus infection is a light-density, nonadherent, HLA-DR$^+$, non-NK, non-B, non-T cell type that represents no more than 1-2% of PBLs (92, 510, 827, 828). The lineage of this cell type is not known, but it can be clearly distinguished from monocyte/macrophages and from dendritic cells on the basis of antigenic and adherence characteristics (92; S. Bandyopadhyay, personal communication). The killing of virus-infected target cells, but not of K562 target cells, by CD16$^+$, HLA-DR$^-$ NK cells has an absolute requirement for the IFN-α-producing HLA-DR$^+$ cells (510, 1022, 1023). The HLA-DR$^+$ cells in contact with virus-infected target cells produce a factor that activates NK cells, as shown by supernatant fluid transfer or by separating the HLA-DR$^+$ cells and CD16$^+$ NK cells by filters (510). In both cases the NK cell-activating activity is completely blocked by anti-IFN-α antibodies (510). The same antibodies do not prevent lysis of virus-infected cells by total PBLs (510), as was also described in other studies (1016, 1018). However, anti-IFN-α antibodies efficiently inhibit cytotoxicity on virus-infected target cells when the cultures are rocked to reduce cellular interactions or when the number of HLA-DR$^+$ cells in the culture, normally in large excess, is reduced to the minimal concentration required for efficient cytotoxicity (S. Bandyopadhyay, personal communication). These results strongly support the original hypothesis that activation of NK cells by IFN-α is the major and most efficient mechanism responsible for the enhanced lysis of virus-infected target cells by PBLs *in vitro*. However, other mechanisms, such as the direct interaction of NK cells with IFN-α-producing cells or with viral glycoproteins on the target cell surface are likely to play a role in the activation of NK cells.

B. NK Cells in Bacterial and Parasitic Infection

A role for microbial infection in the maturation and activity of NK cells is supported by data showing earlier maturation and increased NK cell activity in newborn mice or piglets maintained in normal colony conditions versus germ-free animals (276, 1024).

Infection of mice with various bacterial strains such as *Listeria monocytogenes* or *Chlamydia trachomatis* induces a systemic increase of NK cell activity, which peaks at day 3 and returns to normal levels at day 7 in the bone marrow and spleen, but remains increased for more than 10 days in the peripheral blood and peritoneal exudate (1025, 1026). However, no direct role in the resistance to *L. monocytogenes* infection could be attributed to NK cells, because the increase in NK cell cytotoxicity was observed in strains of mice genetically resistant or sensitive to *L. monocytogenes* infection (1025, 1027) and treatment of mice with ^{89}Sr to suppress NK cell activity had no effect on this infection (1028).

It is possible that the major role of NK cells against bacterial infection is the production of lymphokines such as IFN-γ, GM-CSF, TNF, and macrophage-chemotactic factor that activate other effector cells of nonadaptive resistance. However, in some experimental conditions *in vitro* NK cells have been shown to be able to directly lyse extracellular bacteria (1029, 1030) or cells infected with intracellular bacteria, such as *Shigella flexneri*-infected HeLa cells (1031), or monocytes infected with *Legionella pneumophila* (1032) or *Mycobacterium avium* (1033). Recently, purified CD16$^+$, NKH-1/Leu-19$^+$ NK cells have been shown to kill both gram-positive and -negative bacteria. This bactericidal activity is mediated at least in part by an extracellular mechanism involving soluble factors (1034). Treatment of human lymphocytes *in vitro* with fixed *Shigella* or *Salmonella* bacteria induces activation of NK cells and production of both IFN-α and -γ (1035-1037). The cytotoxic NK cells generated have been shown to be Leu-19$^+$ cells, most of which bear the CD16 antigen but not T cell antigens (1036, 1037). The activation of NK cells is also observed using bacteria concentrations too low to induce IFN production, suggesting the possibility that the enhancement of cytotoxicity is due to a direct effect of the bacteria on NK cells and that it is not mediated by IFN (1036). Elimination of CD16$^+$ NK cells from the lymphocyte preparation almost completely eliminates the production of IFN (1037), suggesting that at least the IFN-γ induced in PBL preparations by bacteria is produced by NK cells. Similar results indicating the production of IFN-γ by NK cells have been obtained with murine NK cells induced by *L. pneumophila* (1038). Bacterial lipopolysaccharide (LPS) from *Salmonella* fails to induce IFN production or NK cell activation and inhibits NK cell stimulation by the fixed bacteria (1035, 1037). However, LPS from *Escherichia coli, Pseudomonas aeruginosa*, or human periodontal pathogens plays a central role in the activation of NK cells by these bacteria (1039, 1040). LPS is internalized in NK cells and induces an increase in cytotoxic as well as phagocytic activities for opsonized bacteria. This functional effect is accompanied

by morphological changes (e.g., dilatation of the intracellular membrane compartment, formation of tubuloreticular inclusions, and increase in acid phosphatase activity) that are reminiscent of those induced by IFN (1040).

Soluble streptococcal products also activate NK cells and induce IFN-γ production (1041). The increase in cytotoxicity induced by these products is mostly mediated by the IFN-γ (1041). Streptococcal preparation OK432, often used in therapeutic trials, also has strong IFN-inducing and NK cell-enhancing activity (1042, 1043). *In vivo*, OK432 stimulates an increase in NK cell generation in the bone marrow and the appearance of proliferating NK cells in spleen (1043).

Murine and human NK cells can bind and inhibit growth of fungi such as *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, and *Coccidioides immitis* (1044–1046). There is some evidence that NK cells play a role in controlling cryptococcal infection. Beige mice are less resistant to *C. neoformans* infection than are their normal heterozygous littermates (1047), and the ability of cyclophosphamide-treated mice to clear the fungus is restored by adoptive transfer of normal spleen cells but not by that of spleen cells treated with anti-asialo-GM_1 serum (1048). *In vivo* treatment with anti-asialo-GM_1 serum or anti-NK-1.1 monoclonal antibodies reduces the lung clearance of intravenously injected *C. neoformans*, but has no effect on long-term survival of the mice (1049). The colonization of *C. neoformans* to lung, spleen, and brain after infection via the respiratory route is also not affected by *in vivo* depletion of NK cells (1049). Thus, NK cells have activity against *C. neoformans* but do not appear to play an essential role in the defense against infection in the normal host, although their activity might be required in the immune-compromised host.

Candida albicans enhances NK cell activity (1050) and induces production of TNF from NK cells and monocytes (1051). However, NK cells do not kill *C. albicans*, although an excess of *C. albicans* blocks NK cell lysis of K562 cells (1052). NK cells may participate in the defense against *C. albicans* infection by secreting TNF, IFN-γ, or other lymphokines that activate the fungicidal activity of neutrophils and macrophages (1053).

Few studies have been published on the possible role of NK cells in the defense against protozoa. *In vivo* infection with *Toxoplasma* or *Plasmodium* is associated with increased NK cell activity (1054, 1055). A role for NK cells in the defense against these pathogens is suggested by the shorter survival time of *Plasmodium berghei*-infected beige mice than normal mice (1056), and by the ability of murine NK cells to lyse *Toxoplasma gondii in vitro* (1057). NK cell activation in acute infection and depression in chronic infection has been demonstrated in mice infected with

Leishmania (1058). Studies employing beige mice, split-dose irradiation, and adoptive transfer of an NK cell clone have suggested a possible role for NK cells in the clearance of *Leishmania* from spleen and liver (1059). NK cells show little spontaneous cytotoxicity for trypanosomes, but efficient ADCC (1060). Trypanosomes are not efficiently lysed by NK cell granular pore-forming proteins, but are sensitive to a Ca^{2+}-independent granule lytic protein (1060).

X. NK Cells and Adaptive Immunity

A. Immunoregulatory Role of NK Cells on B Cell Response

Moretta *et al.* (1061) originally showed that E-rosetting $Fc\gamma R^+$ lymphocytes, after interaction with immune complexes, suppress the polyclonal B cell differentiation induced by pokeweed mitogen (PWM). E-rosetting $Fc\gamma R^+$ cells are now known to be almost exclusively $CD2^+$, $CD16^+$ NK cells. Lobo (1062) showed that non-E-rosetting $Fc\gamma R^+$ cells, probably corresponding to the $CD2^-$ subset of NK cells, spontaneously enhanced PWM-induced B cell differentiation but suppressed it after interaction with immune complexes, providing the first experimental evidence that NK cells might have both enhancing and suppressive effects on B cell response. The effect of NK cells on PWM-induced B cell differentiation was attributed to an indirect effect of NK cells on helper T cells rather than to a direct effect on B cells (1061, 1063). A murine NK cell clone was shown to inhibit B cell response both *in vivo* and *in vitro* (1064). Although some studies have shown that B cells at different stages of activation are sensitive to the lytic effect of NK cells (1064–1066), this sensitivity has not always been confirmed (1067) and most evidence from different experimental systems suggests that B cell lysis by NK cells during an immune response is not a major mechanism by which NK cells modulate B cell response.

The human suppressor cells activated by immune complexes were further identified as NK cells by reactivity with antibody HNK-1/Leu-7: the ER^-, HNK-1/Leu-7^+ cells were more suppressive than were ER^+, HNK-1/Leu-7^+ cells, suggesting a role for NK cells rather than for HNK-1/Leu-7^+ T cells (1068). Suppressor ability of NK cells [identified by HNK-1/Leu-7 (1069) or CD16 (1070) expression] was shown to be activated by IFN-α, whereas a subset of suppressor T cells was activated by PWM (1069). Within the $CD8^+$ cells, $CD8^+$, $CD11b^-$ T cells required the presence of $CD4^+$, $2H4^+$ suppressor/inducer cells to suppress PWM-induced B cell differentiation, whereas $CD8^+$, $CD11b^+$ cells (mostly NK cells) did not require the inducer cell population and, unlike

suppressor T cells, were enhanced in their suppressor effect by IL-2 (1071). In addition to their suppressive action on PWM-induced B cell differentiation, human NK cells suppress ongoing Ig synthesis by *in vivo* activated B lymphoblasts secreting anti-tetanus toxoid antibodies (1069), by EBV-induced B cells (1070), and by certain lymphoblastoid cell lines (1072), although they enhance Ig synthesis by other cell lines (1072). These results suggest that NK cells can interact directly with B cells and modulate their activity. The possible involvement of at least some of the mechanisms of the lytic process in suppression of the B cell response by NK cells is suggested by the competitive effect of a low number of K562 target cells (1069), by the ability of antibody 13.1 (anti-gp200) to inhibit both NK cell-mediated cytotoxicity and Ig synthesis suppression (1073), and by the possible involvement in both mechanisms of the B cell TfR, as indicated by the reversal of the inhibitory effect in the presence of iron ions (1074).

Normal human and murine bone marrow contains potent natural suppressor cells. The human natural suppressor cells are HNK-1/Leu-7^+ and devoid of T cell markers, and possibly include mature or immature NK cells (1075). In murine bone marrow natural suppressor cells are radiation-sensitive Qa-2^+ cells that express markers of mature NK cells only after stimulation with IL-2-containing conditioned medium, suggesting that they might be proliferating pre-NK cells (1076). The same cells appear to be responsible for "veto" activity, i.e., they are able to specifically prevent the generation of CTLs directed against their own MHC antigens (1076).

The presence of HNK-1/Leu-7^+ cells in the germinal center of lymphoid follicles was originally considered as morphological evidence for the involvement of NK cells in the immune response, but these cells have now been identified as $CD4^+$ T cells without NK cell cytotoxic activity (154, 1077).

Treatment of mice with anti-NK-1.1 serum before or at the time of immunization with either T cell-dependent or -independent antigens induces a severalfold increase in the number of antibody-forming cells in the spleen (1066, 1078). Injection of antiserum 8 hours after immunization has no effect, suggesting that the suppressive effect of NK cells is mostly at the induction phase of the B cell response (1066, 1078). *In vivo* treatment with anti-asialo-GM_1 serum enhances the IgM response to different types of pneumococcal polysaccharides, in both adult and weanling mice (1079). Because weanling mice do not have cytotoxic NK cells but do have a normal number of cells with phenotypic characteristics of NK cells, it is possible that NK cells are able to suppress the B cell response even when their cytotoxic activity is absent, because

of either immaturity or suppression (1079). In all of these experimental systems, in which depletion of NK cells enhances the B cell response, *in vivo* activation of NK cells with poly I:C is accompanied by inhibition of the B cell response (1066, 1079).

In contrast with the studies showing an effect of NK cells mostly on the induction phase of the B cell response, Abruzzo and Rowley (1080) proposed that NK cells have an homeostatic effect on the antibody response by both inhibiting induction and promoting termination of the primary IgM response *in vivo* when administered in animals up to 3 days after immunization. Poly I:C or IFN treatments resulted in inhibition or termination of the IgM response (1081) and in activation of NK cells able to suppress B cell responses *in vitro*. The involvement of NK cells in the normal homeostasis of immunity was suggested by the fact that immunization itself induced NK cell activation with a peak at 2–3 days using T cell-independent antigens and at 4–5 days using T cell-dependent antigens (1081). The suppressive effect of NK cells is not antigen specific and the immunized mice showed a generalized IgM hyporesponsiveness to unrelated antigens, corresponding to the peaks of NK cell activation induced by the primary immunization (1081). This hyporesponsiveness was abolished in mice in which NK cell activity was depressed by treatment with pristane, by growth of a transplantable fibrosarcoma, or by repeated injection of poly I:C (1081). The NK cells in this experimental model have been shown to act by suppressing the accessory capacity of dendritic cells exposed to antigens (1080). This effect on dendritic cells and the suppressive effect of NK cells on the B cell response was shown to be mediated by Thy-1$^-$ NK cells but not by Thy-1$^+$ NK cells (1082).

Evidence for an enhancing effect of NK cells on the B cell response was provided by studies showing that NK cells, in the absence of T cells, support the *in vitro* antigen-specific murine B cell response in T cell-replacing, factor-dependent systems or upon *in vitro* stimulation with T cell-independent antigens (1083, 1084). In these systems, the enhancing effect of NK cells was mediated by the production of IFN-γ (1083, 1084).

The supernatant of unstimulated purified NKH-1/Leu-19$^+$ human NK cells was found to enhance ongoing IgE, IgG, and IgA syntheses from appropriate B cell lines, without increasing cell proliferation (1085). This late-acting B cell differentiation activity was produced by CD3$^-$, but not CD3$^+$, NKH-1/Leu-19$^+$ cells and was found to be different from that of other known lymphokines with partially overlapping activities (1085). PBLs from patients after T cell-depleted allogeneic bone marrow transplantation contain an expanded and

activated NK cell population; these PBLs spontaneously produce IL-2, IFN-γ, and B cell differentiation factor and provide non-antigen-specific help for Ig production by autologous B cells, more consistently observed after treatment of the cells with anti-CD2 antibodies (1086, 1087). Anti-CD2 antibodies, which moderately inhibit NK cell cytotoxicity, might prevent a suppressive or toxic effect of NK cells on B cells, although anti-CD18 antibodies, which suppress cytotoxicity of K562 target cells more efficiently than do anti-CD2 antibodies, have no effect on B cell response (1086).

Purified human $CD3^-$, NKH-1/Leu-19^+ NK cells are induced to express FcϵR or FcαR on 10-20% of the cells, when exposed to IgE-anti-IgE or IgA-anti-IgA immune complexes, respectively. Supernatant fluids enhanced IgE or IgA syntheses from Ig-secreting B cell lines in an isotype-specific fashion without increasing proliferation (1088). Thus, NK cells, but not $CD3^+$ T cells, express isotype-specific FcR and produce differentiation factors for that isotype after interaction with specific Ig isotypes in complexes (1088). NK cells incubated with IgE complexes react with antibody anti-CD23, specific for the low-affinity FcϵR; fragments of the FcϵR are able to become soluble IgE-binding factors able to regulate IgE synthesis (1089)

Human NK cell clones can produce B cell differentiation factors that induce Ig production from B cell lines and can induce Ig synthesis from purified B cells only when the NK cell clones are cocultured with the B cells (1090). TNF and IFN-γ are among the factors produced by NK cell clones and by non-MHC-restricted CTL clones that enhance *in vitro* antibody formation (R. F. Schmidt, personal communication).

B. Immunoregulatory Role of NK Cells on T Cell Response

Treatment of mice with anti-asialo-GM_1 serum prevents the induction of alloantigen-specific CTLs *in vivo* by immunization with allogeneic spleen cells (1091, 1092). The same requirement for asialo-GM_1^+ cells in the generation *in vitro* of alloantigen-specific CTLs was shown in one study (1092), whereas many other studies (1091, 1093-1095) have shown that asialo-GM_1^+ murine NK cells or $CD16^+$ human NK cells suppress *in vitro* T lymphocyte proliferation or generation of CTLs and that this suppressive effect is enhanced by IFN-α.

NK cells suppress CTL generation and T cell proliferation in allogeneic or autologous mixed-leukocyte culture by suppressing or eliminating dendritic cells that have interacted with antigen (1094, 1096). In secondary mixed-leukocyte cultures, which are efficiently stimulated by either dendritic cells or macrophages, NK cells suppress only the stimulation by

dendritic cells (1097). On the other hand, studies using Percoll-purified LGL preparations have suggested that subsets of human LGLs provide accessory cell functions for T cell proliferation in autologous and allogeneic mixed-leukocyte cultures (1098) and for *in vitro* generation of virus-specific CTLs (1099). However, those studies did not exclude contamination of the LGL preparation with accessory cells such as dendritic cells or the HLA-DR$^+$ IFN-α-producing cells that copurify with LGLs in Percoll gradient. Purified human NK cells are unable to function as antigen-processing cells, although they can present alloantigens after *in vitro* activation with phytohemagglutinin and IL-2 (115). Purified CD16$^+$ human NK cells are also unable to function as accessory cells in various types of T cell-proliferative responses (1100, 1101), although they did support phytohemagglutinin-induced T cell proliferation to a very low extent (1100) and, in the presence of a source of accessory cells, enhanced a mixed-leukocyte reaction (1101).

XI. Anti-Tumor Activity of NK Cells

A. Studies of Experimental Animals

In order for NK cells to play a role in the control of tumor growth, they require the ability to interact with and destroy syngeneic tumor cells or to indirectly activate other adaptive and nonadaptive mechanisms of antitumor resistance. The ability of NK cells to lyse syngeneic cells was proven using transformed cell lines as the target (56), but fresh tumor cells are almost insensitive to NK cell lysis. Studies in which NK cells were enriched and/or activated with IFN or IL-2 showed that allogeneic and autologous fresh tumor cells are sensitive to NK cell-mediated cytotoxicity (37, 79, 776). However, NK cells are not specifically cytotoxic for tumor or transformed cells, and normal cells, e.g., fibroblasts, may be as sensitive or more sensitive to NK cell lysis than are tumor cells (34, 56). The *in vivo* existence of NK cytotoxic cells with a possible function in the surveillance against tumors suggests the importance of *in vivo* regulatory mechanisms to recruit and activate NK cells locally, in analogy with other nonadaptive mechanisms of defense of the organism (34).

In experimental animals the *in vivo* effect of NK cells against tumors was investigated by evaluating long-term growth of tumors (1102), metastasis formation (1103), and short-term elimination of radiolabeled tumor cells from the whole animal or from certain organs (e.g., lungs) (1104, 1105). The experimental protocols used involved analysis of the correlation of NK cell activity and tumor resistance (373, 1106), the use of NK cell-deficient mice (e.g., beige mice) (1107, 1108), or the use of

experimental procedures able to enhance (e.g., treatment with IFN or IFN-inducing substances) (1109–1111) or depress NK cell activity. The latter was achieved by the use of ^{89}Sr (442), split-dose irradiation (453), anti-asialo-GM_1 antiserum (222, 1112), anti-NK cell alloantisera (1113, 1114), anti-NK-1.1 monoclonal antibodies (1115), and anti-IFN antisera (1116). Altogether, these experiments have clearly shown that NK cells are effective *in vivo* and can destroy tumor cells. Transplanted NK cell-sensitive tumors and experimental tumor metastasis can be inhibited by NK cells. The direct role of NK cells in the prevention of metastasis formation was confirmed by reconstitution experiments in which formation of metastasis in NK cell-depleted animals was prevented by adoptive transfer of purified NK cells (1103, 1117) or cloned cell lines with NK activity (1118). However, the evidence for an effective role of NK cells in resistance to spontaneously arising neoplastic cells is much less compelling (1119).

Metastasis often advances by hematogenous spread; the presence, in the blood, of NK cells with cytotoxic activity that can be up-regulated may allow them to lyse tumor cells present in the circulation before these cells colonize to form metastasis. The experiments of *in vivo* clearance of intravenously injected tumor cells, especially when clearance from the lung is measured, mostly measure intravasal destruction of tumor cells, because NK cell-mediated effects are observed before appreciable extravasation of the tumor cells occurs starting at 4 hours (1120). The demonstration that NK cells can eliminate tumor cells in the circulation does not exclude, however, the possibility that prevention of metastasis takes place also at the tissue level. An extravasal antimetastatic effect of NK cells in the lung and the liver was demonstrated using mice treated sequentially with MVE-2 and anti-asialo-GM_1 antiserum, which have increased NK cell activity in both the lung and the liver but depressed circulating NK cells. In these mice metastasis formation was suppressed, suggesting that organ-associated extravasal NK cell activity is a possible mechanism for the antimetastatic therapeutic effects of *in vivo* treatment with NK cell-activating substances (1121).

IL-2-activated lymphocytes (i.e., LAK cells) suppress metastasis formation. The role of NK cells in this activity was determined (1122) by comparing the effect of unfractionated rat LAK cells with that of enriched IL-2-stimulated NK cells obtained by the plastic adherence method (101). The enriched NK cell preparation in combination with IL-2, compared to unfractionated LAK cells, demonstrated a dramatic and superior antimetastatic effect both at liver and lung levels and significantly prolonged survival of the host after treatment (1122).

B. Studies of Cancer Patients

The role of NK cells in the defense against tumors in humans has been the subject of hundreds of papers, but a relationship between NK cell activity and tumor progression has been difficult to establish (1123). The major obstacle to these studies has been the high variability of NK cell number and activity among control healthy donors and the difficulty of a careful quantitation of the results. The difficulties in the quantitative evaluation of NK cell activity and the criteria to be used in order to obtain statistically interpretable data have been reviewed in Section II. The data presented in most studies are the result of experiments that have been performed in a limited number of patients, lack appropriate healthy controls, and do not apply acceptable criteria of quantitation and standardization. The studies in which the phenotype of NK cells has been analyzed often failed to use appropriate reagents and, in most cases, the number of NK cells was expressed only as a proportion of total lymphocytes and not as an absolute concentration of cells. Many reports should therefore be considered anecdotal and do not add much to our knowledge of NK cell functions *in vivo*.

In patients with advanced cancer NK cell cytotoxic activity is usually depressed (1124–1128); this depression appears to be secondary to tumor invasion and due either to interaction of NK cells with tumor cells or to the presence of suppressor cells (746–749). Pross and Baines (1123) reported the analysis of data from the first 307 patients in a study of a total of 1600 randomly chosen cancer patients. The study was performed using monocyte-depleted PBLs in an overnight assay using K562 target cells and applying careful criteria of quantitation and standardization of the assay (53). Each donor was tested in repeated assays (median, 3). Randomly chosen control healthy donors, patients with no evidence of disease, and patients with local disease had comparable cytotoxic activity; patients with metastatic disease and, more so, patients with advanced metastases, displayed significantly lowered NK cell cytotoxic activity (1123). However, the actual differences (0.82 ± 0.09 for advanced metastases versus 1.05 ± 0.07 for patients with no evidence of disease) were not marked, and certainly were not as high as those reported by the same authors (1127) for patients with liver metastases or by others using, in most cases, unfractionated mononuclear cells (1124, 1125, 1128).

The depression of NK cell activity in cancer patients is probably due to several different mechanisms, reflecting the complexity of NK cell regulation *in vivo*. Competition or inactivation by tumor cells, reduced

number of NK cells, reduced responsiveness to IFN or IL-2, ability to produce IFN or IL-2, presence of suppressor cells (including monocyte/macrophages acting through release of prostaglandins), presence of inhibitory substances such as glycoproteins and glycolipids, and other mechanisms have been described as responsible for NK cell depression in cancer patients (reviewed in Ref. 1123).

Most of the studies of NK cell cytotoxic activity in cancer patients have been performed using cells from peripheral blood. It is therefore possible that the decrease in NK cell function or number is in part due to altered circulation of the cells or their sequestration at tumor sites or in draining lymph nodes. However, virtually no NK cell activity is found in malignant effusions or among tumor-infiltrating lymphocytes (747, 1129–1131). The lack of NK cell activity at tumor sites could be due in part to an *in situ* inhibition of NK cell activity, because in some studies (1132–1135) functional cytotoxic NK cells have been enriched from ascites fluid and tumor-infiltrating lymphocytes using Percoll gradient fractionation. Highly cytotoxic $CD2^+$, $CD3^-$, $CD16^+$ cells have been grown in IL-2-containing medium from the ascitic fluid or pleural effusions of patients with advanced ovarian or metastatic breast cancer (1136). NK cell activity was demonstrated in breast tumor draining lymph nodes, whereas it was almost absent in normal lymph nodes (572); however, NK cell activity was suppressed in the lymph nodes more proximal to the tumor and/or with tumor infiltration (572), indicating that both alteration of NK cell localization and *in situ* suppression takes place in cancer patients.

The regulation of NK cell activity in patients with hematopoietic tumors is somewhat different from that observed in patients with solid tumors. Patients with preleukemia or myelodysplastic syndrome have generally reduced NK cell activity (1137–1141). The number of phenotypically identifiable NK cells is, however, normal in most patients and defects in the ability of patients' cells to produce IFN-α or to respond to IFN-α have been reported (1138, 1140). The alteration in the bone marrow environment in these patients is probably responsible for deficient production/differentiation of NK cells, analogously to the situation in 17β-estradiol-treated mice in which noncytotoxic NK-1.1$^+$ NK precursor cells are found (210). A depression of NK cell activity is also observed in patients with acute or chronic leukemia; B cell and myeloid chronic leukemia patients often present a significant proportion of cells with the $CD3^+$, $CD16^+$ phenotype and non-MHC-restricted cytotoxicity (1142, 1143). Cells with this phenotype are rare or absent in healthy donors (135). In patients with pure RBC aplasia associated with B cell chronic lymphocytic leukemia, $CD3^+$, $CD16^+$ cells have been shown to suppress

RBC colony formation *in vitro* and have been proposed to be responsible for the *in vivo* erythropoietic defect (1142, 1144; reviewed in Ref. 1145).

If NK cells play a role in surveillance against malignancies, low NK cell activity should have a prognostic value in determining the risk of developing tumors. Patients with genetic diseases such as CHS or X-LPD, with a primary and secondary depression of NK cell activity, respectively, have a high probability of developing an LPD. In these cases, the etiology of the disorder is probably viral and the role of NK cells may reflect their antiviral, rather than their antitumor, activity (C. Lopez, as quoted in Ref. 1146). In familial melanoma, relatives of the patients, who have an increased risk of developing the tumors, also showed a depressed NK cell cytotoxic activity, suggesting a possible role of NK cells resistance to tumor growth (1147). Unlike patients with other solid tumors, those with primary noninvasive melanoma have low NK cell activity (1147, 1148). Strayer *et al.* (1149) reported NK cell cytotoxicity lower than controls in family members of patients with a higher incidence of tumors and observed that NK cell activity varied inversely with the number of family members with cancer. However, in another study of 155 women at high relative risk for breast cancer (1150), no difference in NK cell activity was found compared to normal controls, with the exception of women with benign breast syndrome, who had slightly elevated NK cell cytotoxicity, possibly because of systemic hormonal changes. Because NK cell activity in healthy donors is variable and the disease itself affects NK cell activity, it is still unknown whether NK cells really have any role in tumor surveillance despite many years of investigation for a possible relationship between low versus high NK cell activity and the probability of developing primary tumors (1151). Only when studies of an extremely large number of patients have been performed, using quantitative and standardized methods, will a possible significant correlation (or lack of correlation) between NK cell cytotoxic activity and tumor development be obtained.

Some information is, however, available on the prognostic value of NK cell cytotoxic activity for the probability of developing metastasis in patients with primary tumors. The recurrence of distant melanoma metastases has been found to be significantly lower in patients with high NK cell cytotoxicity than in those with low activity (1148). Schantz *et al.* (1152) indicated a strong inverse correlation between high NK cell activity and formation of metastasis in patients with head and neck cancer. Pross (1146, 1153), in a 14-year ongoing comparative study of NK cell activity and clinical course in patients with solid tumors, reported a definitive trend correlating high NK cell activity with increased survival time, but this did not quite reach significance ($p < 0.06$; $n = 430$). In those

patients who have been tested multiple times while metastasis free and who subsequently develop metastasis, the correlation between average NK cell activity and time from diagnosis to metastasis was significant ($p < 0.029$; $n = 91$). By contrast, in patients who were disease free (as opposed to metastasis free) at the time of NK cell testing, NK cell activity had no prognostic significance (1147).

XII. Alterations of Human NK Cell Number and Function in Other Pathological Conditions

Numerous studies have been published evaluating the cytotoxic activity or, more rarely, the number of human NK cells in almost any pathological condition. Alterations of NK cell activity, most often a decrease in cytotoxicity, have been reported in patients with different types of disease. Unfortunately, as discussed in the case of cancer patients, most of these studies are performed on a limited number of patients, lack adequate controls, and do not use standardized quantitative methods for NK cell analysis. In the previous sections many of these studies, relevant for the understanding of regulation and function of NK cells, have been reviewed. In this section, other pathological conditions in which NK cells might play a role or in which NK cells are functionally altered will be briefly reviewed. In the interpretation of these studies it is important to consider that, with few exceptions, it is not possible to determine whether the alteration in NK cell activity is primary or secondary to the pathological condition or to the therapy used.

Decreased NK cell-mediated cytotoxicity is generally observed in connective tissue disorders, particularly in systemic lupus erythematosus (SLE), rheumatoid arthritis, and Sjögren's syndrome (reviewed in Ref. 1154). Peripheral blood mononuclear cells from a high percentage of SLE patients have a reduced ability to produce IFN-α *in vitro*, but most patients have measurable circulating IFN-α, suggesting that the defect of NK cell activity might be secondary to continuous exposure to IFN-α *in vivo* (1155). Because of the predominantly suppressive effect of NK cells on B cell activation, the defect in NK cell activity in these patients could favor the activation of B cells producing autoreactive antibodies. This possibility is supported by the observations in mice carrying the autosomal recessive *lpr* gene. These mice spontaneously develop an SLE-like syndrome, accompanied by profoundly decreased NK cell activity in spleen and peripheral blood (1156) but by elevated hepatic NK cell activity (1157). The spontaneous decrease in NK cell activity in *lpr* mice was observed to be associated with an increased autologous plaque-forming B cell

(APFC) response (1156). The APFC response was diminished when NK cell activity was increased with poly I:C treatment, whereas ablation of NK cells with anti-asialo-GM_1 antisera before poly I:C treatment increased the APFC response (1156). The massive T cell proliferation associated with autoimmune disease in *lpr* mice was shown to be similarly regulated by NK cell activity. Neonatal thymectomy increased NK cell activity and retarded the development of lymphoproliferation and autoantibody formation, whereas thymectomized mice treated with anti-asialo-GM_1 antisera developed LPD and splenomegaly (1158). (NZB $\times$ NZW)F_1 mice also spontaneously develop an autoimmune disease resembling SLE. In these animals, however, progression of the autoimmune disease is accompanied by development of a high level of natural killing (1159). Suppression of NK cells *in vivo* using ^{89}Sr reduces both the autoimmunity and the pathologic lesions of SLE, suggesting that in (NZB $\times$ NZW)F_1 mice, unlike in *lpr* mice, NK cells play a role in the acceleration of autoimmunity (1159).

In diabetic *BB/W* rats a role for NK cells in the destruction of islet cells has been suggested on the basis of (1) increased NK cell activity in diabetic or diabetes-prone rats in comparison to diabetes-resistant rats, (2) ability of NK cells from *BB/W* rats to lyse *in vitro* dispersed islet cells, and (3) prevention of diabetes when NK cells are eliminated by *in vivo* treatment with anti-CD8 (OX8) antibodies (1160–1162). In Type I diabetes patients, however, NK cell-mediated cytotoxic activity against K562 cells is lower than in normal controls (1163–1165). The low NK cell activity in these patients is probably genetically determined because nondiabetic identical twins of the patients have been reported to have low NK cell activity (1165). The cytotoxicity of PBLs from active diabetic patients against dispersed islets was, however, higher than that of PBLs from healthy individuals or patients in remission, suggesting the possibility that subsets of NK cells might be differentially regulated in diabetes (1164). The exact nature of the cells cytotoxic for the islets and the mechanism of cytotoxicity remain, however, to be determined.

Multiple sclerosis patients with acute remitting or chronic progressive disease have been shown by numerous investigators to have reduced NK cell-mediated cytotoxicity and ability to produce both IFN-α and IFN-γ (reviewed in Ref. 1166), although in other studies a normal NK cell activity has been reported (56, 1167). Because a similar MHC-linked genetic control has been suggested for both low NK cell activity and multiple sclerosis (56, 356), it is possible that the NK cell defect in the patients is genetically determined and has relevance in the etiopathogenesis of multiple sclerosis, a disease of probable viral origin.

However, the association of low NK cell activity with an active status of the disease (1166) may suggest that the defect in NK cell activity is secondary and irrelevant for the etiopathogenesis of multiple sclerosis.

Alteration of NK cell activity has been described following organ (e.g., kidney) transplantation. In general, a depression of circulating NK cell activity was observed for up to 2 years following transplantation, but an increase in NK cell cytotoxicity was reported during rejection episodes (1168, 1169). These alterations of NK cell cytotoxicity are probably secondary to the immunosuppressive therapy and to the response of the host adaptive immune system against the graft. In experimental animals cytotoxic NK cells have been shown to infiltrate sponge matrix grafts or kidney graft at an early time of rejection (peak at day 4) and to disappear at a later time, when CTLs appear (1170, 1171). *In vivo* treatment with anti-asialo-GM_1 antisera abrogated NK cell activity and delayed appearance of CTLs (1170): these results suggest that NK cells might directly participate in graft rejection and may act as accessory cells for CTL generation. There is, however, no evidence that NK cells are involved as effector cells in organ rejection following clinical transplantation (1172).

Depressed NK cell cytotoxicity is one of the many immunological defects observed in patients with AIDS, AIDS-related complex, or lymphoadenopathy syndrome (1173-1176). NK cells from these patients are characterized by a defect at the postbinding stage of lysis (1173-1176). After binding to target cells, they fail to polarize tubulin (1176) and to release NKCF (1174). In addition to the defect in the lytic mechanism, a selective depletion of lymphocytes with the phenotype of NK cells was observed among human immunodeficiency virus (HIV)-positive infected patients (1177). The decrease in NK cells was both relative and absolute; the $CD8^+$, $CD16^+$, NKH-1/Leu-19^+ subset of NK cells was much more decreased than was the $CD8^-$, $CD16^+$, NKH-1^+ subset (1177). Because no functional differences between the $CD8^+$ and $CD8^-$ subsets of NK cells have been described, the significance of the selective depletion of one subset only is difficult to interpret.

Cytolytic activity of human mononuclear PBLs from healthy donors cultured in IL-2 was abrogated after 3 days of cultures by *in vitro* infection with HIV (1178). HIV antigens were expressed on infected cells after only 14 days of culture. At this time all $CD4^+$ cells and most $CD16^+$ cells expressed HIV antigens, suggesting that HIV might also have a tropism for NK cells (1178). The possibility that HIV might infect NK cells directly is an interesting finding that deserves further investigation. PBLs from AIDS patients have a reduced expression of IFN-α, but not IFN-γ, receptors (1179). The down-modulation of the IFN-α receptor may be due to the continuous exposure to the circulating serum acid-labile IFN-α observed in the patients; continuous exposure to IFN-α

might also be responsible for the inactivation of NK cell cytotoxicity. The peripheral blood mononuclear cells from AIDS patients are severely deficient, compared to cells from healthy individuals, in their ability to produce IFN-α in response to HSV-1-infected fibroblasts (1180). The defect in IFN-α production has been reported to correlate with susceptibility to opportunistic infection better than the decrease in NK cell cytotoxicity (1180). Thesa data suggest absence or a functional deficiency of the IFN-α-producing HLA-DR$^+$ cells, which are necessary accessory cells for NK cell cytotoxicity against virus-infected cells.

HIV proteins, or fragments of them, might be responsible for the NK cell defect as well as for other immunological defects in AIDS patients. Two synthetic peptides constructed on the basis of two sequences of the HIV transmembrane gp41 (1181), and one peptide with homology to a region of p15E conserved among numerous retroviruses (1182), are potent suppressors of several immunological responses, including NK cell-mediated cytotoxicity. These peptides inhibit lysis by interfering with postbinding lytic mechanisms (1181, 1182). It is interesting to note that NK cells, treated with these peptides, are able to bind to target cells, but fail to polarize the Golgi apparatus toward the target cells, thus appearing to be blocked at the same postbinding stage described as the NK cells from AIDS patients with defective cytotoxic ability.

AIDS patients have very complex immunological and hematological defects, and it is difficult at this time to define the role of decreased NK cell activity in the pathogenesis of the disease. However, because NK cells have antimicrobial and antitumor activities and are able to affect hematopoiesis, it is of theoretical and practical importance to determine whether NK cells in AIDS patients play roles in preventing opportunistic infections or sarcoma development and in determining the hematopoietic dysfunctions observed in these patients.

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